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# Perivascular sirolimus-delivery system

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ABSTRACT

Autologous vein grafts are often used for treating damaged vessels, e.g. arteriovenous fistulas or arterial bypass conduits. Veins have a different histological structure from arteries, which often leads to intimal hyperplasia and graft restenosis. The aim of this study was to develop a perivascular sirolimus-delivery system that would release the antiproliferative drug sirolimus in a controlled manner. Polyester Mesh I was coated with purasorb, i.e. a copolymer of L-lactide and ε-caprolactone, with dissolved sirolimus; Mesh II was coated with two copolymer layers; the layer with dissolved sirolimus was overlaid with pure purasorb. This arrangement allowed sirolimus to be released for 6 and 4 weeks, for Mesh I and Mesh II, respectively. Mesh II released sirolimus more homogeneously, without the initial burst effect during the first week. However, the cumulative release curve was steeper at later time points than the curve for Mesh I. Both meshes inhibited proliferation of rat vascular smooth muscle cells during 14-day culture *in vitro* and preserved excellent cell viability. Newly developed sirolimus-releasing perivascular meshes are promising devices for preventing autologous graft restenosis.



## 1. Introduction

Damage or stenosis of a vessel caused either by injury or by some pathological processes, e.g. atherosclerosis and thrombosis, must often be treated by replacing the vessel with an autologous graft, mostly vein. Four-year patency of an autologous saphenous vein was achieved in 40–70% of treated patients (Taylor et al., 1990; Conklin et al., 2002). Intimal thickening, however, often occurs in veins used as arteriovenous fistulas or arterial bypass conduits, due to the different structure of the vein wall and the arterial wall. In addition, the layer of endothelial cells (EC) in the autologous graft is often damaged during surgery. The restoration of a new EC lining usually lasts several weeks, which results in relatively long-term direct exposure of vascular smooth muscle cells (VSMC) to the blood stream. Platelets and macrophages from the blood start to adhere to the denuded luminal surface of the graft, which is followed by platelet aggregation and release of growth and migratory-promoting factors from these cells (Liuzzo et al., 2005). Growth factors released from platelets, EC, and VSMC, e.g. PDGF, stimulate phenotypic modulation of VSMC from contractile to synthetic phenotype, which is characterised by excessive proliferation and migration of VSMC, and their extracellular matrix production. This results in intimal hyperplasia and graft stenosis (Liuzzo et al., 2005).

Drug-eluting stents were the first local anti-proliferative drug-delivery systems introduced in interventional cardiology. Commercially available stents (e.g. BX Velocity™, Cypher™, Cordis, Johnson & Johnson) and also newly developed sirolimus-eluting stents have been proved to reduce neo-intimal formation in vessels (Mehilli et al., 2008). The restenosis rate was reduced from 20–30% to 1–3% after 1 year (Morice et al., 2002). Drug-eluting stents, however, cause increased mechanical strain on a vessel or damage to the endothelium and thrombosis (Colombo and Iakovou,

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2004). The damaged vein graft is re-endothelialized within several weeks after implantation. During this time an antiproliferative drug should be released from a suitable drug delivery system.

Mechanical strain on the endothelium can be avoided by stent-free drug-delivery systems, e.g. periadventitial or perivascular films, gels, or cuffs. These systems can be advantageously based on a degradable synthetic polymer loaded with a drug, which is continuously released during the polymer degradation. This degradation is usually hydrolytic and not mediated by cells, thus the system can be spontaneously removed from the patient's organism. However, the kinetics of both polymer degradation and drug release should be adjusted to the period necessary for regeneration of endothelial cell layer damaged by the surgery; i.e. at least a few weeks. From this point of view, promising results have been obtained with PEG-Cys-NO hydrogels loaded with S-nitrosothiols, i.e. nitric oxide precursors. When applied perivascularly, these hydrogels generated NO for up to 50 days and inhibited VSMC proliferation, while the proliferation of endothelial cells was increased. This system also inhibited platelet adhesion *in vitro* and reduced neointima formation in a rat carotid balloon injury model at 14 days by approximately 80% compared to controls (Lipke and West, 2005). However, nitric oxide precursors, although some of them occur naturally *in vivo*, have not yet been approved for clinical use.

Another drug with antiproliferative effects, which is widely used in current clinical practice, is sirolimus, also known as rapamycin. Sirolimus is a macrocyclic lactone antibiotic produced by *Streptomyces hygroscopicus*, often used as an antiproliferative agent in drug-eluting intravascular stents. Sirolimus binds to the FK binding protein complex (FKBP12), which subsequently binds to the mammalian target of rapamycin (mTOR) (Daemen and Serruys, 2007). Interaction with mTOR prevents phosphorylation of p70S6 kinase, 4E-BP1, and indirectly also of other proteins involved in transcription, translation, and cell cycle control and progression (Vignot et al., 2005). Sirolimus exhibited a dose-dependent reduction in intimal hyperplasia using 60–200 μg sirolimus-coated stents in the rabbit model. In the porcine model, sirolimus-eluting stents, and stents releasing both sirolimus and dexamethasone reduced the neointimal area compared to bare metal stents after 28 days. This resulted in a 50% decrease of in-stent restenosis (Suzuki et al., 2001).

Sirolimus has also been tested for its potential use in a perivascular drug delivery system. Non-constrictive perivascular poly(ε-caprolactone) (PCL) cuffs releasing paclitaxel or rapamycin allowed dose-dependent sustained drug release for 3 weeks. Their application reduced intimal thickening of the treated femoral arteries by 75% and 76%, respectively (Pires et al., 2005). Pluronic gel containing 200 μg of sirolimus reduced intimal hyperplasia by 41% after 6 weeks (Schachner et al., 2004). Films made of poly(lactic-co-glycolic acid) (PLGA) and PLGA blended with methoxypolyethylene glycol (MePEG) loaded with paclitaxel and wrapped around the injured carotid artery degraded after 28 days in rats (Jackson et al., 2004). Owen et al. (2010) investigated injectable terpolymer ReGel made of, i.e. poly(lactic-co-glycolic acid)–polyethylene glycol–poly(lactic-co-glycolic acid) (PLGA–PEG–PLGA) containing sirolimus (2.5 mg/ml in 2 ml of gel) either in the form of a suspension or a solution *in vivo* on pigs. However, in this case, the gel had to be replenished with the drug at 1, 2, and 3 weeks post-operatively. In addition, most of the above-mentioned systems were based on materials with relatively weak mechanical properties, requiring special handling (manipulation) during the surgical procedure or prone to move away from the desired therapeutic position.

The aim of the study was to develop a periadventitial drug delivery system consisting of a polyester silk mesh, coated with a degradable copolymer purasorb loaded with sirolimus. The polyester mesh was expected to give a stable mechanical support to the sirolimus-releasing system, to facilitate and accelerate wrapping the system around the vascular graft, and to prevent migration of the system out of the periadventitial position. The release of sirolimus onto aqueous and non-aqueous media was then measured by UHPLC, and the antiproliferative effects of the system were tested in cultures of rat aortic smooth muscle cells.

## 2. Materials and methods

### 2.1. Materials

A knitted polyester silk mesh (CHS 50, PES Mesh) was obtained from VUP Joint-Stock Company, Brno, CR. Purasorb PLC 7015, a grade copolymer of L-lactide and ε-caprolactone (70/30 molar ratio, inherent viscosity midpoint of 1.5 dl/g; semicrystalline, without residual monomers) was purchased from PURAC Biomaterials. Acetonitrile (ACN; 99.95%, Biosolve), methanol (99.95%, Chromapur GG) and dichlormethane (min. 99%, Chromapur GG) were purchased from Chromservis (Prague, Czech Republic). HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (USA). Sirolimus (Rapamycin from Streptomyces, Cat. No. R0395) was obtained from Sigma–Aldrich (Germany).

### 2.2. Sample preparation

#### 2.2.1. Mesh impregnation

The mesh is made from yarns. A yarn of about 90 μm across is formed by polyester fibres 17.5 μm in diameter. Purasorb penetrated into the gaps among the fibres in the yarn during mesh coating. The solutions used for the coating were as follows: *solution 1*: 5.2 mg of sirolimus, 36.4 mg of purasorb in 1 ml of chlorbenzen–ethanol (1.75:1 v/v); *solution 2*: 10.4 mg of sirolimus, 36.4 mg of purasorb in 1 ml of chlorbenzen–ethanol (1.75:1 v/v); *solution 3*: 36.4 mg of purasorb in 1 ml chlorbenzen–ethanol (1.75:1 v/v).

#### 2.2.2. Homogeneous coating of Mesh I

The mesh was dip-coated with solution 1 and dried (30 min), and then coated quantitatively (i.e. with the whole remaining amount of the solution) for the second time with solution 1 and then dried. The impregnated mesh contained 0.14 mg sirolimus homogeneously distributed in 0.98 mg purasorb per 1 $cm^2$.

#### 2.2.3. Gradient coating of Mesh II

The polyester mesh was dip-coated with solution 2 and dried (30 min), and then quantitatively overlaid with solution 3 and dried. The total amount of 0.14 mg sirolimus in 0.98 mg purasorb per 1 $cm^2$ in the impregnated mesh was the same as that after homogeneous coating; however, the sirolimus concentration was expected to be higher inside the yarns than near their surface.

#### 2.2.4. Purasorb Mesh

The polyester mesh was coated with solution 3 and dried, and then overlaid with solution 3 and dried. This mesh was coated with sirolimus-free purasorb, and served as a reference sample.

### 2.3. Sample incubation

Mesh I and Mesh II were cut into pieces 0.5 $cm^2$ and 1 $cm^2$ in area, and were incubated in 5 ml of phosphate-buffered saline (PBS) per $cm^2$ of the mesh at 37 °C on a shaker. The PBS was changed daily. For the analyses, the samples were removed after 0, 1, 4, 7, 9, 11, 14, 17, 21, 28, 35, and 42 days of incubation in PBS. For each time interval, 3–8 samples of the mesh were used.

### 2.4. Stability of sirolimus

The stability of sirolimus in PBS was assessed at 37 °C, −20 °C, and −75 °C at two concentrations of 50 and 750 ng/ml after 0, 24, 48 and 168 h. The samples were 100 times pre-concentrated by extraction to methanol using solid phase extraction (HLB OASIS 3cc, Waters) and were analyzed by Ultra High Performance Liquid Chromatography (UHPLC).

The stability of sirolimus in methanol was assessed at 25 °C and −75 °C at three concentration levels 0.125, 5.0, and 75.0 μg/ml. The samples stored at 25 °C and −20 °C were analyzed after 0, 2, 24, and 48 h, whereas the samples stored at −75 °C were analyzed after 0, 2, 24, 48 h, and 5, 15, 30, and 60 days.

The stability of sirolimus in dichlormethane was assessed at 25 °C at three concentration levels 0.125, 5.0, and 75.0 μg/ml after 0, 5, 10, and 30 min.

### 2.5. Sirolimus extraction and UHPLC analysis

After incubation of Mesh I and Mesh II in PBS buffer, the meshes were removed and dried at room temperature. Three mililiters of dichlormethane were added to the mesh for 10 min in order to wash purasorb and sirolimus out of the mesh. The mesh was removed and dichlormethane was evaporated to dryness. Sirolimus was then reconstituted in 1 ml of methanol, centrifuged 5 min at 13,000 rpm in order to eliminate undissolved purasorb, and the supernatant was immediately analyzed. If the sirolimus concentration during the incubation experiment reached a limit of quantification (LOQ), sirolimus was reconstituted in 0.1 ml of methanol, and the obtained amount of sirolimus was divided by 10. Analyses were performed on the Acquity UHPLC system (Waters) equipped with a 2996 PDA detector operating in the range from 194 to 600 nm. Chromatograms of sirolimus analyses were extracted and subsequently quantified at 278 nm. The data was processed using Empower 2 software (Waters).

Samples were injected on a Waters BEH C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm); the mobile phase consisted of solvent A, 10% ACN, and solvent B, ACN; linear gradient elution (min/%B): 0/60, 1.5/100, 2/100; flow rate, 0.4 ml $min^{-1}$; column temperature, 50 °C; injection volume, 5 μl. Each analysis was followed by an equilibration step (0.5 min).

### 2.6. Calculation of the remaining weight of sirolimus

The remaining weight of sirolimus on meshes $X$ (μg/$cm^2$) was calculated as follows:

$$X = C_{\mathrm{UHPLC}} \times \frac{3.97}{m} \quad (1)$$

where $C_{\mathrm{UHPLC}}$ (μg $ml^{-1}$) represents the concentration of sirolimus extracted from one piece of mesh and measured by UHPLC, $m$ represents the weight of analyzed piece of sirolimus and purasorb-free mesh measured after extraction (see Section 2.5), and constant 3.97 represents the weight of 1 $cm^2$ of sirolimus and purasorb-free mesh.

### 2.7. Partial validation of the sirolimus UHPLC method

The sirolimus quantification method was partially validated. The calibration curve over the linear range from 3.125 to 100 μg $ml^{-1}$ was determined using methanol solutions of sirolimus at concentration levels of 100.0, 50.0, 25.0, 12.5, 6.3, and 3.1 μg $ml^{-1}$. LOQ was determined as the lowest point of the calibration curves with precision (expressed as relative standard deviation, RSD%) less than 20% and accuracy of 80–120% in six replicates.

### 2.8. Scanning electron microscopy

The pure PES Mesh and Mesh I were used without any processing and after 2 and 6 weeks of incubation in PBS at 37 °C and drying in a vacuum oven. The samples coated with 2 nm platinum in an SCD 050 Sputter Coater (Balzers Union AG, Balzers, Liechtenstein) were observed using a Quanta™ Scanning Electron Microscope 200F (FEI Czech Republic, s.r.o.).

### 2.9. Size-exclusion chromatography

The samples of Purasorb Mesh were incubated in PBS at 37 °C for 0, 2, 4, and 6 weeks. In addition, the originally purchased polymer purasorb was evaluated without any incubation. The molar mass distribution of purasorb dissolved at a concentration of 2 mg/ml in tetrahydrofuran (THF) and dimethylformamid (DMF) (10:1) was measured by size-exclusion chromatography (SEC) in THF/DMF carried out on a Waters SEC modular system using coupled PLgel $10^3$ Å, 10 μm (7.5 mm × 600 mm), and PLgel MIXED C (7.5 mm × 600 mm) columns (Polymer Laboratories, Ltd.) with a Waters 410 RI detector.

### 2.10. Cells and culture conditions

Polyester meshes (PES Mesh), meshes coated with purasorb (Purasorb Mesh) and meshes coated with purasorb mixed with sirolimus (Mesh I and Mesh II) were sterilized by ethylene oxide. After sterilization, the samples were stored in a vacuum oven at 35 °C for 4 weeks in order to remove the rest of organic solvents used during preparation. Polystyrene dishes (24-well test plate, TPP, Switzerland; well diameter 1.5 cm) were seeded with VSMC derived from the intima-media complex of the thoracic aorta of 8-week-old male Wistar SPF rats by the explantation method (Bačáková et al., 2002), and were used in passage 5–10. VSMC were seeded at an initial number of 16,000 cells/well (i.e. population density of about 9000 cells/$cm^2$) into 1.5 ml Dulbecco-modified Eagle Minimum Essential Medium (DMEM; Sigma, St. Louis, MO, U.S.A.; Cat. No. D5648), supplemented with 10% of fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and 40 μg/ml of gentamicin (LEK, Ljubljana, Slovenia). Twenty-four hours after cell seeding, the samples (PES Mesh or Purasorb Mesh or Mesh I or Mesh II, each 1 $cm^2$) were added into the wells of the culture plates. Pure polystyrene (PS) without added meshes was used as a control. The cells were cultured for zero, two, seven and 14 days after adding the meshes at 37 °C in a humidified air atmosphere containing 5% of $CO_2$. For each experimental group and time interval, 3 samples were used.

### 2.11. Cell viability and number

Cell viability was measured using a LIVE-DEAD Viability/Cytotoxicity Kit (Invitrogen). VSMC were washed with PBS, and incubated with calcein AM (1 μM) and ethidium homodimer-1 (2 μM) for 15 min. The number of living cells (stained green) and dead cells (stained red) were counted from micrographs that were taken under an Olympus IX 71 epifluorescence microscope with a DP 71 digital camera. For each sample and time interval, 30–45 homogenously distributed microscopic fields were used.

### 2.12. Statistical analysis

The quantitative data was presented as mean ± SEM (standard error of mean) or mean ± RSD (relative standard deviation, Table 1). The statistical analyses were performed using SigmaStat (Jandel Corporation, U.S.A.). Multiple comparison procedures were made by the ANOVA, Student–Newman–Keuls method. The value $p \leq 0.05$

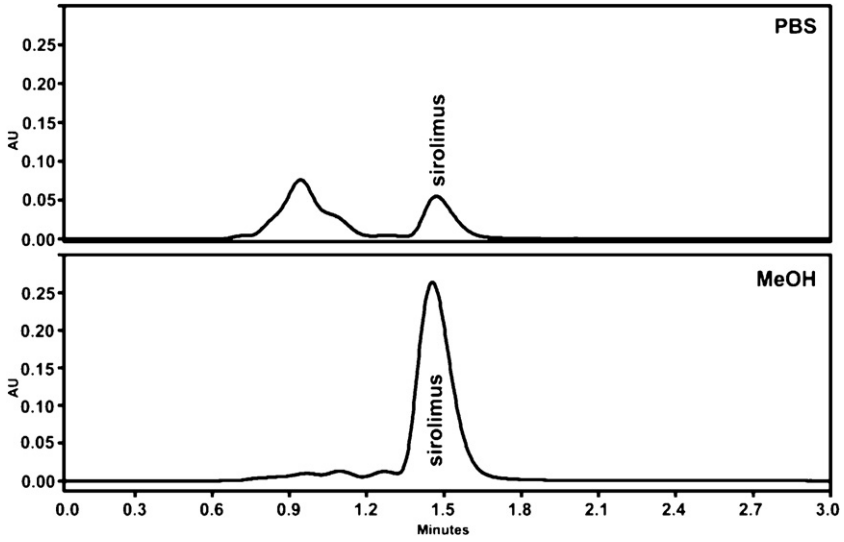


**Fig. 1.** Chromatogram of sirolimus after incubation at 37 °C for 24 h in PBS and methanol (the concentration of sirolimus was 25 μg/ml). Chromatographic conditions: UPLC column Acquity BEH C18 (50 mm × 2.1 mm I.D., particle size 1.7 μm); mobile phase A, 10% acetonitrile, and B, acetonitrile; linear gradient elution (min/%B): 0/60, 1.5/100, 2/100; flow rate 0.4 ml $min^{-1}$; column temperature, 50 °C; injection volume, 5 μl; UV max 278 nm; retention time of sirolimus 1.48 min.

was considered significant. For statistical evaluation of the cumulative release of sirolimus we used STATGRAPHICS Centurion XV software (StatPoint, U.S.A.) and the statistical literature (ČSN ISO 2602). The confidence intervals for the estimated mean values and the confidence limits for the plot of the fitted models were calculated at a confidence level of 95%.

## 3. Results

### 3.1. Partial validation of the sirolimus UHPLC method

The original rapid UHPLC method for determining sirolimus was developed; for the parameters, see Section 2.5. Under the conditions that were developed, baseline separation of sirolimus without any interference was obtained. The sirolimus peak retention time was 1.48 min (see Fig. 1). The calibration curve was linear with regression equations of $y = 3.32 \times 10^4 + 3.49 \times 10^4$ and determination coefficient of 0.999. LOQ was determined as 3.125 with μg $ml^{-1}$ with precision (RSD) of 2.2% and accuracy of 102.4% ($n = 6$). The recovery of the extraction method was 101.1% with RSD of 8.6%.

**Table 1**
Stability of sirolimus dissolved in PBS at 37 °C, −20 °C, and −75 °C. Residues of sirolimus (%) are presented as the mean of the measured concentrations ± $L$; where $L$ represents the confidence interval (95%) and was calculated as follows: $L = R \times K_n$, where $R$ is the difference between the lowest and highest measured concentration, $K_n$ is constant for $n$ replicates at a confidence level of 95%; $n = 4$ and $K_4 = 0.72$.

| Temperature | Time (h) | Residue of sirolimus (%) for relevant concentrations | |
|---|---|---|---|
| | | 50 (ng/ml) | 750 (ng/ml) |
| | 0 | 100 (%) | 100 (%) |
| 37 °C | 24 | 20.9 ± 7.5 | 26.5 ± 4.1 |
| | 48 | 12.5 ± 5.2 | 8.5 ± 2.6 |
| | 168 | (Under detection limit) | (Under detection limit) |
| −20 °C | 24 | 45.6 ± 4.8 | 67.8 ± 5.2 |
| | 48 | 21.0 ± 8.2 | 39.1 ± 4.8 |
| | 168 | (Under detection limit) | (Under detection limit) |
| −75 °C | 24 | 44.6 ± 4.7 | 72.2 ± 5.6 |
| | 48 | 27.0 ± 6.5 | 71.5 ± 5.3 |
| | 168 | 26.8 ± 6.6 | 69.2 ± 5.1 |

### 3.2. Stability of sirolimus in water and methanol

It was found out that sirolimus is not stable in aqueous media including PBS (Fig. 1 and Table 1). Table 1 shows that after 24 h in PBS, sirolimus degraded by more than 70%. It was therefore

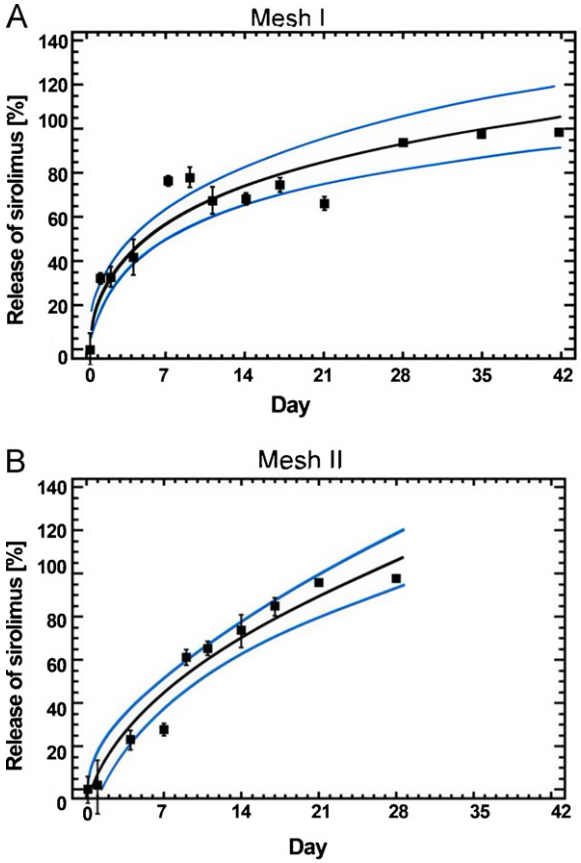


**Fig. 2.** Accumulated release of sirolimus from the coated polyester meshes, i.e. Mesh I and Mesh II. The data is presented as the means ± SEM, blue curves represent two-sided confidence intervals (at a confidence level of 95%). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

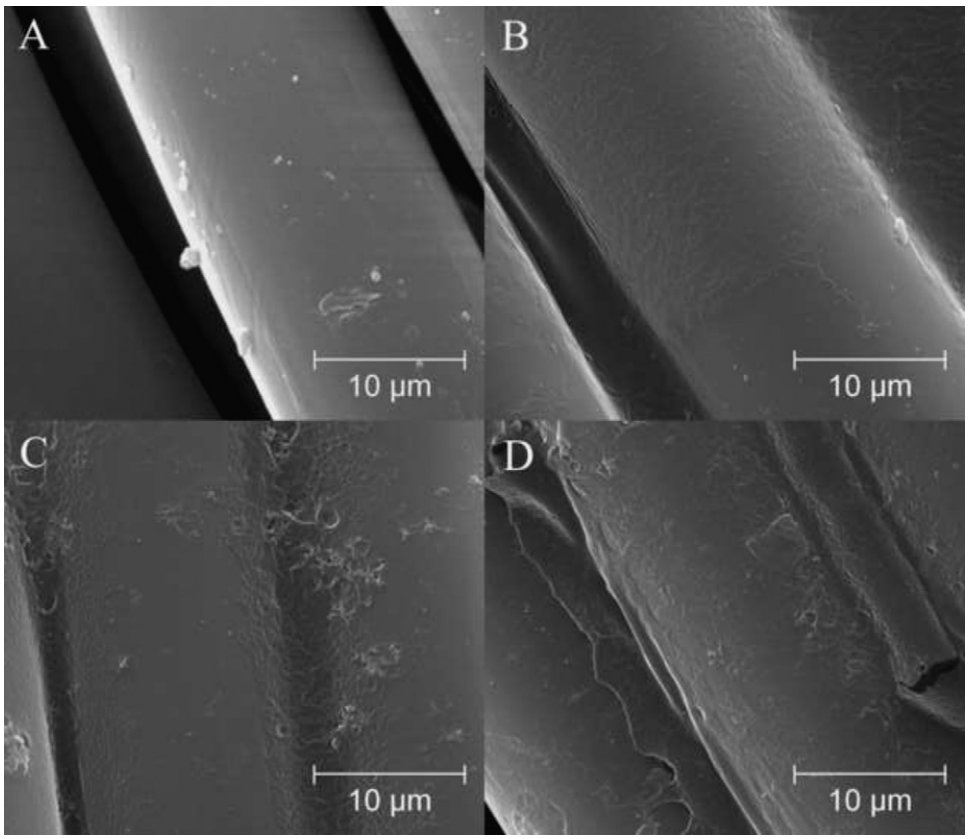


**Fig. 3.** Micrographs of the PES Mesh (A), Mesh I before incubation in PBS (B), Mesh I after 2 weeks (C), and 6 weeks (D) of incubation in PBS, taken under scanning electron microscopy, magn. 2000×, 30.0 kV.

not possible to determine sirolimus released into PBS directly. Instead, the sirolimus retained in the mesh was assayed, and the amount of released sirolimus was calculated. For this purpose, an investigation was made of the stability of methanol and dichlormethane, which were used for extracting sirolimus from the meshes. The methanol solution of sirolimus at 25 °C is stable for 2 days, which is sufficient for the sample preparation and analysis. This solution is stable at −75 °C for 60 days, which enables storage of sirolimus stock solutions (data not shown). Dichlormethane was found to be suitable for extraction of sirolimus, because sirolimus in dichlormethane is stable for at least 30 min (data not shown).

### 3.3. Cumulative release of sirolimus from Mesh I and Mesh II

Mesh I consisted of PES Mesh coated with purasorb layer with homogenously dissolved sirolimus. Mesh II was PES Mesh coated with sirolimus-containing purasorb with a higher concentration of sirolimus, and on top there was a layer of pure purasorb. This arrangement of the drug-delivery systems substantially influenced the sirolimus release. Mesh I released the entire amount of sirolimus after 6 weeks, Mesh II after 4 weeks of incubation in PBS (Fig. 2). As regression models, Square root-*Y* logarithmic-*X* $[4.57331 + 1.52937 \times \ln(\text{day})]^2$ and Square root-*X* $[-16.4822 + 23.2462 \times \text{sqrt}(\text{day})]$ were used for Mesh I and Mesh II, respectively; the correlations obtained were 0.9627 and 0.9712 for Mesh I and Mesh II, respectively. An initial burst of sirolimus release from Mesh I was observed during the first week of its incubation. On day 11, however, the percentage of released sirolimus was equal for both meshes, i.e. 67.7% and 65.5% for Meshes I and II, respectively. Mesh II released sirolimus more homogeneously, without the initial burst effect during the first week, but the curve was steeper at later time points than for Mesh I. This shortened the time needed for total sirolimus release from Mesh II to 4 weeks.

### 3.4. Scanning electron microscopy

A copolymer film was found on the surface of the fibres, and it also bridges gaps between the fibres. Erosion of the film was visible after 2 weeks of incubation with PBS. A broken film was observed after 6 weeks.

### 3.5. Molecular weight

The molecular weight of purasorb from the Purasorb Meshes after 2, 4, and 6 weeks of incubation in PBS was similar to its weight before incubation (Fig. 4). The values of $M_w$ 117,250 and $M_n$ 85,000 were determined for the original polymer supplied by PURAC. Pura-

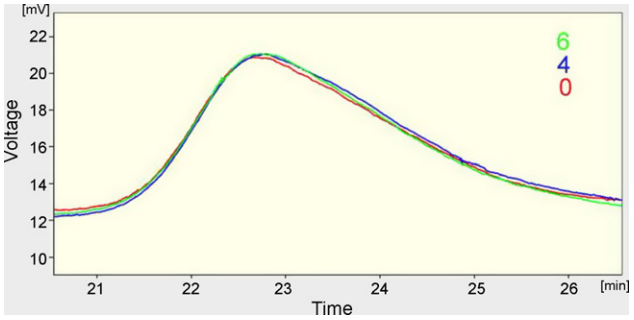


**Fig. 4.** Chromatogram of purasorb after 0 (red), 4 (blue) and 6 (green) weeks of incubation in phosphate-buffered saline at 37 °C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

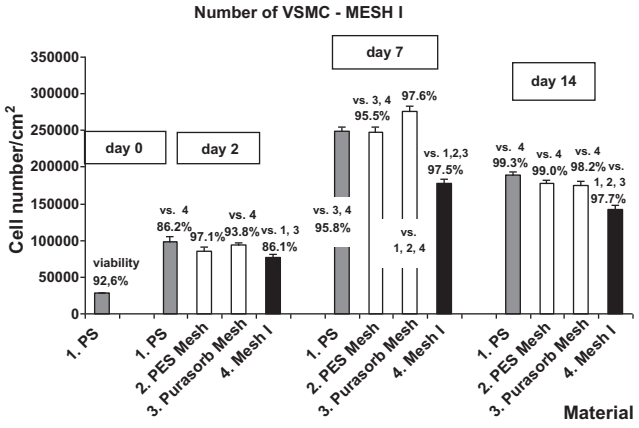


**Fig. 5.** Number and viability of vascular smooth muscle cells on a pure polystyrene culture dish (PS), on PS with a polyester mesh (PES Mesh), a purasorb-coated PES Mesh (Purasorb Mesh) or a sirolimus-containing PES Mesh (Mesh I) on day 0, 2, 7 and 14 after adding the meshes into the cultures. $p$-Value <0.05 is considered significant in comparison with the sample of the same number and the day of culture.

sorb solutions obtained by soaking coated meshes in THF/DMF were analyzed in the same way by SEC. No differences in the molecular mass distribution were observed in the samples of originally supplied purasorb, polymer dissolved from intact coated meshes, and polymer dissolved from coated meshes incubated with PBS for 1, 2, 4, and 6 weeks in PBS and then dried.

### 3.6. Cell number and viability

The viability of VSMC was in the range of 86.2–99.4%, and this viability was similar on Mesh I and Mesh II and on the control samples (Figs. 5 and 6). Sirolimus eluted from Mesh I in an *in vitro* experiment significantly reduced the VSMC number on day 2, 7, and 14. The reduction in the VSMC number was by 21.8%, 28.7%, and 24.8% compared to PS on day 2, 7, and 14, respectively. For Mesh II, VSMC proliferation was reduced by 67.6%, 76.1%, and 60.1% compared to PS on day 2, 7, and 14 after adding the meshes, respectively.

## 4. Discussion

Local drug delivery is assumed to have a substantial effect on preventing vascular restenosis without causing systemic adverse reactions (Pires et al., 2005). It prevents drug degradation before reaching the target, and assures excellent uptake of the drug by the tissue (Golomb et al., 1996). However, a long-term follow-up of antiproliferative drug-eluting intravascular stents, containing, e.g. paclitaxel, sirolimus, or tacrolimus, revealed a wide range of adverse reactions, caused by mechanical and biochemical damage to the vascular wall by the stent. These reactions involved particularly damage to the endothelium, increased platelet aggregation and a thrombogenic response, the presence of focal remnants of residual fibrin deposition, induced apoptosis of VSMC, and insufficient reduction of neointimal hyperplasia at 90 and 180 days in animals, when the drug was completely eluted from the stent (Suzuki et al., 2001). Other important complications are inflammation of the vessel wall and local hypersensitivity reaction against the polymer coating the stent. High-molecular weight PLLA (320,000 Da) used as a coating of the tantalum stent caused lower neo-intimal inflammatory response compared to the low-molecular weight PLLA (80,000 Da) coating (Lincoff et al., 1997). The presence of stents in the vascular lumen often leads to impaired healing of endothelium accompanied by an excessive risk of late thrombosis.

On the other hand, a perivascular wrap prevents mechanical damage to the endothelium layer. The risk of late thrombosis caused by sirolimus released from the perivascular delivery system can be minimized by the distance of the sirolimus source from the endothelial cell layer, and by limiting the time of sirolimus release to the several weeks that are needed for re-endothelialization of the graft. However, in earlier studies, the drug carriers for the perivascular drug delivery system were often constructed in the form of a gel, which allowed them to move (migrate) away from the desired therapeutic position (Owen et al., 2010).

We have developed a perivascular drug delivery system based on a relatively mechanically strong polyester mesh, which is expected to be stably wrapped around the vascular graft. In addition, the mechanical support provided by the meshes can prevent distension of the autologous vein graft placed into arterial position, reduces the increased vessel wall stress and in turn leads to inhibition of VSMC proliferation (Mehta et al., 1998).

This mesh is coated with a degradable copolymer of L-lactide and ε-caprolactone (i.e. purasorb) containing sirolimus. Release from degradable matrices is usually triphasic, contains an initial burst release, a diffusional phase, and a degradation-controlled phase (Venkatraman and Boey, 2007). The kinetics of the drug release depends on the physical and chemical properties of the drug, the choice of a degradable polymer or copolymer, and their arrangement into polymer bilayers or multilayers with and without the drug, and also barriers (Finkelstein et al., 2003).

The degradation of polycaprolactone is within the order of 2–3 years, which is a much slower rate than that of PLA (Gunatillake and Adhikari, 2003). The rate of degradation of polyesters, such as polyglycolide, polylactide, and polycaprolactone, is preferably caused by access of water to the ester bond. Water rapidly plasticizes these polymers, and this in turn leads to mechanical distortion, cracking, pitting and fissure of the polymer in an uncontrolled way (Vert et al., 1994). Paclitaxel release from PLGA/methoxypolyethylene glycol (PLGA/MePEG) films was very slow, with less than 5% of the encapsulated drug being released over 2 weeks. The addition of 30% diblock copolymer composed of PDLLA-block-MePEG to paclitaxel-loaded PLGA films caused a substantial increase (five- to eight-fold) in the release rate of paclitaxel. The water soluble diblock copolymer seemed to enhance the hydrophilicity of the matrix, water uptake, the formation of water-filled channels throughout the matrix, and greatly increased the paclitaxel release rates (Jackson et al., 2004).

The copolymer used in the study, i.e. copolymer of L-lactide and ε-caprolactone (70/30) was of a relatively high $M_W$, i.e.

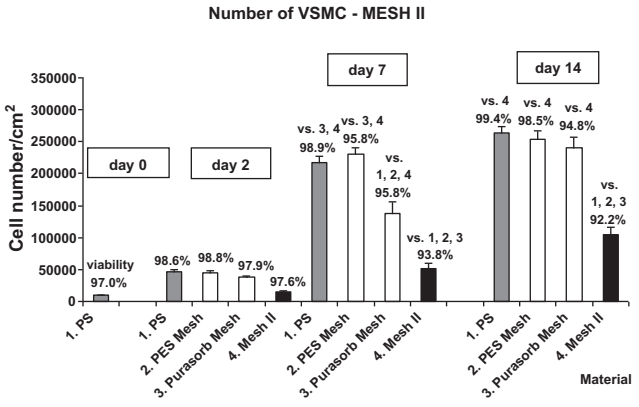


**Fig. 6.** Number and viability of vascular smooth muscle cells on a pure polystyrene culture dish (PS), on PS with a polyester mesh (PES Mesh), a purasorb-coated PES Mesh (Purasorb Mesh) or a sirolimus-containing PES Mesh (Mesh II) on day 0, 2, 7 and 14 after adding the meshes into the culture. $p$-Value <0.05 is considered significant in comparison with the sample of the same number and the day of culture.

117,250 g/mol. After 2 weeks (Fig. 3) and 4 weeks (not shown) of incubation in PBS, we observed only small erosion changes in the copolymer coating; cracks appeared after 6 weeks. No changes in molar mass distribution observable by SEC indicated that there was no hydrolytic degradation of the purasorb polymer chains. Thus, sirolimus was released from the polymer matrix by diffusion rather than by polymer degradation. Mesh I released the total amount of sirolimus after 6 weeks, and Mesh II after 4 weeks. From Mesh I, there was substantial release of the drug in the first week of incubation, while the sirolimus release from Mesh II was more homogenous, without an initial burst release of the drug (Fig. 2). The difference in the kinetics of sirolimus release was caused by the different coating of the two meshes. Mesh I was coated with a homogeneous layer containing sirolimus. Mesh II contained the first layer which was twice as concentrated as that in the Mesh I, and the second upper layer composed of pure copolymer. The upper layer prevented an initial burst effect of sirolimus from Mesh II, due to gradual sirolimus diffusion through the pure copolymer. A similar arrangement, which prevented an initial burst drug release, was used in a study on bilayer rapamycin-eluting stents, coated with two different PLGA layers (Pan et al., 2009). The first PLGA layer (95 kDa, 85/15) contained a substantial amount of the drug (316 μg), while the upper layer was composed of PLGA of low $M_w$ (20 kDa, 75/25) and contained only a small portion of rapamycin (30 μg).

The release of sirolimus differed according to the composition and arrangement of the two matrices. The sirolimus release from Mesh II, being completed after 4 weeks, was faster than the 6-week release from Mesh I. This may have been caused by a higher concentration of sirolimus in the first (i.e. bottom) layer of the Mesh II coating, which may have created a high concentration gradient of sirolimus, supporting drug diffusion into the upper layer and its release. In other studies, the complete drug release has varied, being observed after 15, 28, or even 90 days (Venkatraman and Boey, 2007; Hausleiter et al., 2005; Pires et al., 2005). Perivascular poly(ε-caprolactone) (PCL) cuff released all paclitaxel or rapamycin after 3 weeks (Pires et al., 2005). Films made of poly(lactic-co-glycolic acid) (PLGA) and PLGA blended with methoxypolyethylene glycol (MePEG) loaded with paclitaxel used as perivascular wraps released only 5–20% of the drug after 30 days (Jackson et al., 2004).

Both Mesh I and Mesh II inhibited growth of VSMC in culture for 14 days, although the Mesh II reduced VSMC proliferation more apparently (Figs. 4 and 5). This correlates positively on with the kinetics of sirolimus release, as the same amount of drug (140 μg/cm$^2$) in the Mesh II is released in a shorter time interval of 4 weeks. However, the Mesh I has been releasing sirolimus for 6 weeks which can be useful for *in vivo* studies in the future. In addition, as indicated by staining of VSMC by LIVE-DEAD Viability/Cytotoxicity Kit, the growth of VSMC was inhibited without significant death of these cells, which can be considered as a favourable effect preserving the physiological functions of VSMC, particularly their contractility. On the contrary, treatment with the rapamycin-eluting Pluronic gel, applied into the perivascular spaces of the grafted vein in mice, was associated with an increased apoptosis rate in the vascular wall, including the tunica media (Schachner et al., 2004). However, in this case, the dose of rapamycin was higher than in our system (i.e. 140 μg/cm$^2$ of the mesh), amounting to 200 μg per 0.1 ml of the gel.

In clinically used Cypher$^{TM}$ stents, the total load of sirolimus is in the range of 70–300 μg (Venkatraman and Boey, 2007). Two profiles of drug release from stents have been prepared: fast release profile released the total amount of sirolimus (i.e. 140 μg/cm$^2$) in 15 days, and slow release that needed 90 days for the release of the drug. In our study, we used the same concentration of sirolimus per cm$^2$ in both Mesh I and Mesh II). Bare microporous metal stents without polymer coating were loaded with three concentrations of sirolimus, i.e. 138, 313, and 479 μg/mm$^2$. Complete release was found after 4 weeks with 66% of drug released in the first week (Hausleiter et al., 2005).

A significantly higher amount of sirolimus (2.5 mg/ml in 2 ml of gel) was used in the injectable terpolymer ReGel made of poly(lactic-co-glycolic acid)–polyethylene glycol–poly(lactic-co-glycolic acid) (PLGA–PEG–PLGA) (Owen et al., 2010). An *in vitro* study showed that 65–80% of the drug was released by day 14. For *in vivo* application, the gel should be replenished at 1, 2, and 3 weeks post-operatively. Mean sirolimus concentrations in all tissue sections analyzed at 1 week were found to be below 3 ng/mg of tissue, and were lower at this early time point than the experimentally determined concentration that was required to inhibit the proliferation of cultured smooth muscle cells by 50% ($IC_{50}$ = 5 ng/ml).

## 5. Conclusions

We developed a perivacular system for controlled delivery of antiproliferative drug sirolimus. The perivascular delivery system is intended for wrapping a vein graft after its implantation into an arterial position. The system is based on polyester mesh coated with copolymer of L-lactide and ε-caprolactone with dissolved sirolimus. Two similar formulations of the system were able to release sirolimus within 4 or 6 weeks. The concentrations of sirolimus released from both meshes inhibited proliferation of smooth muscle cells in culture for 14 days. The perivascular sirolimus-delivery system seems to be promising for prevention of intimal hyperplasia and graft restenosis.

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## References

Bačáková, L., Lisá, V., Kubínová, L., Wilhelm, J., Novotná, J., Eckhart, A., Herget, J., 2002. UV light – irradiated collagen III modulates expression of cytoskeletal and surface adhesion molecules in rat aortic smooth muscle cells *in vitro*. Virchows Arch. 440, 50–62.

Colombo, A., Iakovou, I., 2004. Drug-eluting stents: the new gold standard for percutaneous coronary revascularisation. Eur. Heart J. 25, 895–897.

Conklin, B.S., Richter, E.R., Kreutziger, K.L., Zhong, D.-S., Chen, C., 2002. Development and evaluation of a novel decellularized vascular xenograft. Med. Eng. Phys. 24, 173–183.

ČSN ISO 2602 (010231), 1993. Statistical interpretation of tests results. Estimation of mean. Confidence intervals.

Daemen, J., Serruys, P.W., 2007. Drug-eluting stent update 2007: part I: a survey of current and future generation drug-eluting stents: meaningful advances or more of the same? Circulation 116, 316–328.

Finkelstein, A., McClean, D., Kar, S., Takizawa, K., Varghese, K., Baek, N., Park, K., Fishbein, M.C., Maškar, R., Litvack, F., Eigler, N.L., 2003. Local drug delivery via a coronary stent with programmable release pharmacokinetics. Circulation 107, 777–784.

Golomb, G., Fishbein, I., Banai, S., Mishaly, D., Moscovitz, D., Gertz, S.D., Gazit, A., Poradosu, E., Levitzki, A., 1996. Controlled delivery of a tyrphostin inhibits intimal hyperplasia in a rat carotid artery injury model. Atherosclerosis 125, 171–182.

Gunatillake, P.A., Adhikari, R., 2003. Biodegradable synthetic polymers for tissue engineering. Eur. Cell Mater. 5, 1–16.

Hausleiter, J., Kastrati, A., Wessely, R., Dibra, A., Mehilli, J., Schratzenstaller, T., Graf, I., Renke-Gluszko, M., Behnisch, B., Dirschinger, J., Wintermantel, E., Schömig, A., Investigators of the individualizable drug-eluting stent system to abrogate restenosis project, 2005. Prevention of restenosis by a novel drug-eluting stent system with a dose-adjustable, polymer-free, on-site stent coating. Eur. Heart J. 25, 1475–1481.

Jackson, J.K., Smith, J., Letchford, K., Babiuk, K.A., Machan, L., Signore, P., Hunter, W.L., Wang, K., Burt, H.M., 2004. Characterization of perivascular poly(lactic-co-glycolic acid) films containing paclitaxel. Int. J. Pharm. 283, 97–109.

Lincoff, M.A., Furst, J.G., Ellis, S.G., Tuch, R.J., Topol, E.J., 1997. Sustained local delivery of dexamethasone by a novel intravascular eluting stent to prevent restenosis in the porcine coronary injury model. J. Am. Coll. Cardiol. 29, 808–816.

Lipke, E.A., West, J.L., 2005. Localized delivery of nitric oxide from hydrogels inhibits neointima formation in a rat carotid balloon injury model. Acta Biomater. 1, 597–606.

Liuzzo, J.P., Ambrose, J.A., Coppola, J.T., 2005. Sirolimus- and taxol-eluting stents differ towards intimal hyperplasia and re-endothelialization. J. Invasive Cardiol. 17, 497–502.

Mehilli, J., Byrne, R.A., Wieczorek, A., Iijima, R., Schulz, S., Bruskina, O., Pache, J., Wessely, R., Schömig, A., Kastrati, A., 2008. Intracoronary stenting and angiographic restenosis investigators—test efficacy of rapamycin-eluting stents with different polymer coating strategies (ISAR-TEST-3). Randomized trial of three rapamycin-eluting stents with different coating strategies for the reduction of coronary restenosis. Eur. Heart J. 29, 1975–1982.

Mehta, D., George, S.J., Jeremy, J.Y., Izzat, M.B., Southgate, K.M., Bryan, A.J., Newby, A.C., Angelini, G.D., 1998. External stenting reduces long-term medial and neointimal thickening and platelet derived growth factor expressionin space a pig model of arteriovenous bypass grafting. Nat. Med. 4, 235–239.

Morice, M.C., Serruys, P.W., Sousa, J.E., Fajadet, J., Ban Hayashi, E., Perin, M., Colombo, A., Schuler, G., Barragan, P., Guagliumi, G., Molnar, F., Falotico, R., RAVEL Study Group, 2002. Randomized study with the sirolimus-coated Bx velocity balloon-expandable stent in the treatment of patients with de novo native coronary artery lesions. A randomized comparison of a sirolimus-eluting stent with a standard stent for coronary revascularization. New Engl. J. Med. 346, 1773–1780.

Owen, S.C., Li, H., Sanders, W.G., Cheung, A.K., Terry, C.M., 2010. Correlation of tissue drug concentrations with in vivo magnetic resonance images of polymer drug depot around arteriovenous graft. J. Control. Release 146, 23–30.

Pan, C.J., Tang, J.J., Weng, Y.J., Wang, J., Huang, N., 2009. Preparation and in vitro release profiles of drug-eluting controlled biodegradable polymer coating stents. Colloids Surf. B. Biointerfaces 73, 199–206.

Pires, N.M., van der Hoeven, B.L., de Vries, M.R., Havekes, L.M., van Vlijmen, B.J., Hennink, W.E., Quax, P.H., Jukema, J.W., 2005. Local perivascular delivery of anti-restenotic agents from a drug-eluting poly(epsilon-caprolactone) stent cuff. Biomaterials 26, 5386–5394.

Schachner, T., Zou, Y., Oberhuber, A., Tzankov, A., Mairinger, T., Laufer, G., Bonatti, J.O., 2004. Local application of rapamycin inhibits neointimal hyperplasia in experimental vein grafts. Ann. Thorac. Surg. 77, 1580–1585.

Suzuki, T., Kopia, G., Hayashi, S., Bailey, L.R., Llanos, G., Wilensky, R., Klugherz, B.D., Papandreou, G., Narayan, P., Leon, M.B., Yeung, A.C., Tio, F., Tsao, P.S., Falotico, R., Carter, A.J., 2001. Stent-based delivery of sirolimus reduced neointimal formation in a porcine coronary model. Circulation 104, 1188–1193.

Taylor Jr., L.M., Edwards, J.M., Porter, J.M., 1990. Present status of reversed vein bypass grafting: five-year results of a modern series. J. Vasc. Surg. 11, 193–206.

Venkatraman, S., Boey, F., 2007. Release profiles in drug eluting stents: issues and uncertainties. J. Control. Release 120, 149–160.

Vert, M., Mauduit, J., Li, S., 1994. Biodegradation of PLA/GA polymers: increasing complexity. Biomaterials 15, 1209–1213.

Vignot, S., Faivre, S., Aguirre, D., Raymond, E., 2005. mTtor-targeted therapy of cancer with rapamycin derivates. Ann. Oncol. 16, 525–537.