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Vascular and bone cells on polymeric structures for tissue engineering

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ABBREVIATIONS

bFGF	basic fibroblast growth factor
BMP	bone morphogenic protein
BSP	bone sialoprotein
cAMP	cyclic adenosine monophosphate
Cdk2	cyclin-dependent kinase 2
cGMP	cyclic quanosine monophosphate
DMEM	Dulbecco's modified Eagle's medium
EC	endothelial cells
ECM	extracellular-matrix
EGF	epidermal growth factor
ePTFE	expanded polytetrafluoroethylene
FGF-2	fibroblast growth factor-2
GGGRGDS	Gly-Gly-Gly-Arg-Gly-Asp-Ser
GRGDSG	Gly-Arg-Gly-Asp-Ser-Gly
GRGDY	Gly-Arg-Gly-Asp-Tyr
HUVEC	human umbilical vein endothelial cells
IGF-1	insulin-like growth factor-1
IKVAV	Ile-Lys-Val-Ala-Val
IL-1	interleukin
KRSR	Lys-Arg-Ser-Arg
MMPs	metalloproteinases
NO	nitric oxide
PDGF	platelet-derived growth factor
PDLLA	poly(D,L-lactic acid)
PE	polyethylene
PEG	poly(ethylene glycol)
PEEK	poly(ether-ether keton)
PEO	poly(ethylene oxide)
PET	poly(ethylene terephtalate)
PGA	poly(glycolic acid)
PHSRN	Pro-His-Ser-Arg-Asn
PMNs	polymorphonuclear granular leukocytes

PLA	poly(lactic acid)
PLLA	poly(L-lactic acid)
PLGA	poly(lactic-co-glycolic acid)
PPACK	D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone
PTFE	polytetrafluoroethylene
REDV	Arg-Glu-Asp-Val
RGD	Arg-Gly-Asp
$RGDG_{13}PHSRN$	Arg-Gly-Asp-Gly_{13}-Pro-His-Ser-Arg-Asn
RNIAEIIKDI	Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor-α
VEGF	vascular endothelial cell growth factor
VSMC	vascular smooth muscle cells
YIGSR	Tyr-Ile-Gly-Ser-Arg

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1. INTRODUCTION

1. 1. Autologous grafts, allografts and xenografts in vascular and bone surgery

1.1.1. Vascular grafts

Damage or stenosis of a vessel caused by injury or by some pathological processes, e.g. atherosclerosis or thrombosis, must often be treated by replacing it with an autologous or artificial graft. Autologous grafts, mostly veins, have usually given better results in their patency. The four-year patency of an autologous saphenous vein was in the range of 40-70% (Taylor *et al*. 1990, Conklin *et al.* 2002). Autologous grafts are predominantly used for replacing small diameter-vessels, where artificial grafts often fail (Kannan *et al*. 2005).

However, the use of autologous grafts may cause problems. Intimal thickening often occurs in veins used as arteriovenous fistulas or arterial bypass conduits, due to the different structure of the vein and arterial wall. In addition, the layer of endothelial cells (EC) in an autologous graft is often damaged during surgery, and the new EC lining is restored within a few weeks. In an intact vessel, EC control thrombosis, vasomotor tone, smooth muscle proliferation and migration, lipid infiltration, leukocyte adhesion, transmigration and transformation (Nugent and Edelman 2001). Damage to the intima of the autologous graft exposes vascular smooth muscle cells (VSMC) to the blood stream. Platelets and macrophages from the blood start to adhere to the surface. Platelet adhesion is followed by platelet aggregation and the release of growth and migratory-promoting factors, such as PDGF, IL-1, IGF-1, FGF-2, VEGF, and others, or vasoactive amins, e.g. serotonin and thromboxane A_2 (Topol and Serruys 1998; Liuzzo *et al.* 2005).

Fully differentiated VSMC in adult artery exhibit low rates of proliferation or death, and are rarely called upon to migrate or to re-establish vascular integrity (Newby and Zaltsman 2000). The quiescence of VSMC is maintained by heparin sulphate proteoglycans, which interfere with the activation of transcription factors by the protein kinase C-dependent pathway. Compounds that increase cAMP concentration (e.g. prostaglandin E_1 and I_2) or cGMP concentration (NO and atrial natriuretic factors) also inhibit VSMC proliferation (Newby *et al*. 1992; Newby and Zaltsman 2000). Interactions between basement membrane components and cell surface integrins inhibit phenotypic modulation of VSMC i.e., their transition from a contractile phenotype to a synthetic phenotype, characterized by increased migration and growth activity (Thyberg *et al*. 1997; Newby and Zaltsman 2000).

Phenotypic modulation of VSMC means the transition from the so-called “contractile” phenotype to the “synthetic” phenotype of these cells. The contractile phenotype is typical for VSMC in mature and healthy blood vessels, whereas the synthetic phenotype occurs in damaged vessels, and also in VSMC grown in a conventional cell culture system, i.e. in serum-supplemented media and under static conditions. The contractile phenotype of VSMC is characterized by abundant and well-developed contractile filaments containing the alpha-isoform of actin, and the SM1 and SM2 isoforms of the myosin heavy chain and other associated molecules, such as muscle-type of tropomyosin, T-troponin, h-caldesmon, h1-calponin (Hai and Gu 2005; Sung *et al*. 2005). As revealed by transmission electron microscopy, the contractile apparatus fills most of the cell volume, whereas the cellular organels, especially those associated with proteosynthesis, such as the endoplasmic reticulum or Golgi complex, are located at the cell periphery near the cell membrane. Other important markers of the differentiated contractile phenotype of VSMC involve the presence cytoskeletal proteins desmin, vimentin, meta-vinculin and smoothelin, and especially very low or no apparent migratory and proliferation activity (Girjes *et al*. 2002; Quensel *et al*. 2002; Stiebellehner *et al*. 2003; Brinck *et al.* 1997). During phenotypic modulation, VSMC quantitatively lose the above-mentioned specific components of the contractile filaments and cytoskeleton. In addition, these components change qualitatively, i.e. into their non-muscle isoforms (for example, alpha-actin is replaced by the beta-isoform of this protein). The synthetic organels move into the central part of the cells, and increase their number and metabolic activity, which is manifested by enhanced synthesis of various ECM molecules, enzymes, chemoattractants and growth factors, e.g. PDGF-like molecules. As a result, the synthetic VSMC activate their migratory and proliferative potential.

Excessive migration, proliferation and synthetic activity of VSMC is an important complication of vascular surgery, including replacement of damaged vessels by natural and synthetic vascular grafts, because it often leads to restenosis and failure of the graft. Therefore, in the conventional vascular grafts currently used in clinical practice, VSMC have beeen avoided as a component of these grafts. However, it should be taken into account that VSMC are the physiological and most numerous cellular component of the natural vacular wall, and thus their introduction into bioartificial blood vessel prostheses, and control over their phenotype, is a major challenge for advanced tissue engineering.

As for the growth factors released when a vessel is injured, PDGF stimulates both phenotypic change and migration of VSMC from media to intima (Ferns *et al*. 1991). FGF-2 is released from injured EC and underlying ECM, and is a potent autocrine and paracrine

inducer of EC proliferation and migration. Although FGF-2 is a less potent VSMC chemoattractant, it is required for PDGF-directed VSMC migration via a calcium/calmodulin-dependent kinase mechanism (Bilato *et al*. 1995; Liuzzo *et al*. 2005). Endogenous FGF-2 stimulates EC migration and increases the production of protease plasminogen activator, which is an important protease facilitating microvascular and macrovascular EC migration (Sato and Rifkin 1988; Liuzzo *et al*. 2005). In contrast, exposure of intact endothelium to FGF-2 has little effect on EC proliferation (Lindner *et al*. 1990; Liuzzo *et al*. 2005). Phenotypic modulation of VSMC is also accompanied by altered regulation of genes for several extracellular matrix proteins. Increased synthesis of hyaluronic acid is an early event. Basement membrane components, e.g. laminin, are down-regulated, while intersticial matrix components, such as type I collagen, elastin and fibronectin, are greatly increased. The abundance of monomeric rather than polymerized collagen plays an important role in allowing cells to proliferate. However, polymeric collagen induced cell arrest in the G1 phase of the cell cycle, and up-regulated the inhibitors of cdk2 $p27^{Kip1}$ and $p21^{Cip1/Waf1}$ (Koyama *et al*. 1996).

VSMC migration into the intima, their proliferation and phenotypic modification, as well as production and deposition of ECM proteins finally result in intimal hyperplasia and restenosis of the graft. The limited availability of vessels, either arteries or veins, suitable for implantation is another reason for accelerating the effort to develop new biocompatible artificial prostheses.

An allograft, i.e. a vessel from another organism of the same species, has an immunogenic potential, and brings a risk of disease transmission. Allografts of heart valves are often used due to their superior hemodynamics, relatively good resistance to infections and no need for anticoagulation treatment (Bader *et al*. 1998). Xenografts, i.e. grafts from other species, have a strong tissue immunogenic potential. Immune reactions caused by xenografts could be reduced by using acellularized allografts, e.g. in the study with heart valves prepared from detergent acellularized porcine aortic valves that were seeded with human EC (Bader *et al.* 1998). On the other hand, relatively good results were obtained with vascular xenografts made from small intestine submucosa that were implanted in the aorta, carotid and femoral arteries in dogs. After 90 days, the grafts were biocompatible, and good patency rates were observed with a histological structure similar to that of the adjacent vessel (Lantz *et al*. 1993; for a review, see Baguneid *et al*. 2006). Similarly, collagen biomaterial derived from the submucosa of the small intestine and type I collagen was found to be antithrombogenic after a 90-day period of implantation in rabbit aortas (Huynh *et al*. 1999;

Baguneid *et al*. 2006). Also, xenografts made from acellularised porcine aorta were repopulated with human myofibroblasts and endothelial cells from saphenous vein biopsies (Bader *et al.* 2000; Baguneid *et al*. 2006).

Glutaraldehyde-fixed bovine and human umbilical vein grafts have been evaluated extensively, but their use was avoided because of high incidence of aneurysm formation beyond two years after implantation (Dardik *et al*. 1988; Conklin *et al*. 2002).

1.1.2. Bone grafts

Autologous grafts are preferably used for the treatment of bone loss associated with trauma or musculoskeletal diseases. Apart from their immunocompatibility, they are osteoconductive, i.e. their surface supports and directs cell adhesion and bone formation; they are osteoinductive, i.e. they induce differentiation of osteoblast precursors into mature bone-forming cells; they contain cells that take part in bone regeneration. However, their disadvantages are donor site morbidity, and the quantity and quality of bone for harvesting (Parikh 2002). Allografts are more available than autografts, and avoid the need for a second surgical intervention, but can transmit diseases and cause an immune response and in turn lead to graft failure (Kim *et al*. 2006). Unlike autologous grafts, demineralized freeze-dried bone allografts were not able to induce bone formation in dogs in a period of 12 weeks (Becker *et al*. 1995).

1.2. Synthetic polymers for vascular and bone tissue engineering

1.2.1. Non-degradable materials

Artificial vascular grafts of the first generation were constructed as bioinert, i.e. not allowing colonization with cells. This approach was based on the assumption that the basic functions of a graft are not connected with its biological activity. Above all, the vascular graft must carry blood flow, resist dilatory pressures as well as compressive and kinking forces (Hubbell 1999). To achieve these basic goals, no biological activity is necessary. However, the properties of inert grafts are often far from those of an ideal graft.

The ideal vascular graft should be non-toxic, non-thrombogenic, non-immunogenic, strong, compatible with high or low blood flow rates, and have similar viscoeleasticity to native vessels. It should be kink-resistant, available in a variety of lengths and not too expensive (Baguneid *et al*. 2006). It should also allow endothelialization of the inner surface

of the prosthesis. The reason for this is that replacing an inert graft with a bioactive graft can improve the ability of the graft to prevent coagulation, encourage EC attachment and retention, promote capillary infiltration as a source of EC, and prevent excessive VSMC proliferation and collagen matrix synthesis (Hubbell 1999).

There are three possible sources of endothelialization: transanastomotic pannus ingrowth from the native artery itself, transmural tissue ingrowth, and 'fall out' endothelialization by circulating progenitor cell precursors in the blood. However, in humans, the ingrowth of EC deeper than 10 mm from anastomosis is rare (Sales *et al.* 2005; Baguneid *et al*. 2006).

Two inert polymers are most often used in the manufacture of vascular prostheses: PET (Dacron®) and ePTFE (Teflon®). Their bioinertness is based on their relatively high hydrophobia (water drop contact angle about 100^{o}), which hampers the adsorption of cell adhesion-mediating ECM proteins, such as fibronectin or vitronectin, in an appropriate geometrical conformation, and thus disables the accessibility of specific sites on these molecules, e.g. RGD-containing oligopeptides, for cell adhesion receptors, e.g. integrins (Altankov and Groth 1996; for a review, see Bacakova *et al*. 2004). In addition, hydrophobic surfaces preferentially adsorb albumin, which is non-adhesive for cells (for a review, see Bacakova *et al.* 2004).

PET can be woven with small pores or knitted with bigger pores that will improve cell ingrowth, if the prosthesis is converted into a bioartificial graft. PET prostheses have rather good mechanical properties; with tensile strength in the range of 170-180 MPa; the tensile modulus was 14,000 MPa, but they are rather stiff compared to vessels (Kannan *et al*. 2005). PET can be resorbed after 30 years in a human body. It is often used for aortic and peripheral bypass grafts of larger diameter, i.e. above 6 mm. On the other hand, it possesses inherent thrombogenicity, and tends to dilate; to reduce seepage through the prosthesis, it must be pre-clotted with gelatin, collagen, albumin, or blood (Kannan *et al*. 2005, Cardon *et al*. 2000).

Expanded PTFE is nondegradable, antithrombogenic, and has good mechanical properties, i.e. stiffness of 0.5 GPa, tensile strength of 14.0MPa. It is used mainly for lower limb bypass grafts with a diameter of 7-8 mm and for aorto-iliac grafts. Its main disadvantage is high adsorption of proteins or adhesion of platelets. For this reason, it is not suitable for constructing grafts of small diameter. In order to prevent aberrant protein adsorption and cell adhesion, ePTFE has often been modified by binding heparin, heparin on fibrin glue, or carbon coating.

Both PET and PTFE prostheses are rather stiff compared to natural vessels. Mismatches in the mechanical properties of native vessels and graft materials are known to contribute to

the failure of vascular grafts due to chronic anastomotic hyperplasia, i.e. excessive tissue formation in the adjacent arterial wall. In the vessels, suture-line stress concentrations in the native vessels were about 40% higher with stiff Dacron grafts compared to native-vessel grafts (Ballyk *et al*. 1998; Conklin *et al*. 2002). Another important factor implicated in graft failure is a lack of EC lining the inner lumen of the graft (Seifalian *et al*. 2002).

Some other non-degradable polymers have been studied for use in vascular surgery, such as polypropylene and polyurethanes. Polypropylene is a relatively inert polymer, biostable with tensile strength of 400 MPa. It is resistant to hydrolysis, but susceptible to oxidation, so antioxidants must be added during preparation. Polypropylene grafts 4 mm in diameter were found to be patent in 81% in comparison with 69% for Dacron, and 20% for PTFE at 16 months (Kannan *et al*. 2005).

Polyurethanes are a wide group of polymers. Polyurethanes of the first generation have a wide range of stiffness and flexibility according to the structure of their hard and soft segments, but they are degradable *in vivo*. The next generation of polyurethanes is represented by carbonate-based polyurethanes without ester bonds, and these materials are stable. A preliminary study showed faster endothelialization and lower neointima formation in comparison with ePTFE at 6 months (Jeschke *et al*. 1999; Kannan *et al*. 2005). The Corvita® graft from this group, which consists of an inner porous polycarbonate layer and an outer gelatin-heparin surface, showed no changes in diameter 1 year post-implantation. However, polyurethanes composed of some diisocyanates degrade into toxic substances and have a carcinogenic effect. Modified polyurethanes like poly(esterurethane)ureas do not allow good cell adhesion and degrade in an oxidative way (Kannan *et al*. 2005).

When an artificial graft is implanted into an organism, proteins from the blood start to adsorb on its surface, because even grafts that were originally constructed as bioinert (e.g. on the basis of their high hydrophobia) are usually not perfectly antiadhesive. Protein adsorption is followed by the adhesion of several cell types, such as platelets, macrophages, EC, VSMC, lymphocytes, and fibroblasts.

This process gradually results in the formation of three layers, i.e. an inner fibrin layer, an intermediate layer of foreign body giant cells, and an outer connective tissue capsule. In addition, the presence of intimal hyperplasia, i.e. excessive tissue ingrowth through the graft wall, or chronic anastomotic hyperplasia was observed (Walpoth and Bowlin 2005). Intimal hyperplasia is a result of compliance mismatch, that is, mechanical properties of the graft differing from those of the adjacent native vessel, or an abnormal cellular microenvironment.

For example, VSMC interfere continuously with a synthetic material and a non-typical (i.e., not physiological) cellular environment of neighbouring cells and ECM.

The patency of artificial prostheses is therefore rather low, especially the patency of low-diameter prostheses. For examples, PTFE microvessels 1 mm in diameter had patency of about 20%, but all vein grafts in similar settings remained patent (Schmedlen *et al*. 2003; Kannan *et al*. 2005). The rates of thrombosis of grafts less than 6 mm in diameter were greater than 40% (Niklason *et al*. 1999). A possible solution to this problem is to seed the inner graft surface with a confluent layer of autologous EC, creating neointima, which is excellently antithrombogenic.

In bone tissue engineering, polymeric materials have usually been applied in combination with other reinforcing components (e.g., ceramics, carbon fibres, metallic components), because the mechanical properties of the polymers themselves are not suitable for construction of bone implants, especially load-bearing implants. Among non-degradable polymers, PTFE, PET, polypropylene and polyvinyldifluoride have been used as bone replacements (Klee *et al.* 2003). Polytetrafluoroethylene has been applied in the form of membranes to stimulate bone tissue regeneration in oral or craniofacial applications (Suzuki *et al*. 2005).

1.2.2. Degradable polymers for vascular and bone tissue engineering

Both biodegradable and bioabsorbable polymers degrade in the human body and obviate the need for repeated surgery. Biodegradation refers to the enzymatic degradation of polymers *in vivo*, while bioabsorbability means that polymers degrade or dissolve into smaller fragments due to chemicals in the human body (Kannan *et al.* 2005; Griffith 2000). These materials serve as a temporary scaffold for the vessel or bone before they are replaced by ingrowing tissue. The tissue ingrowth should be balanced by the rate of degradation of the polymer.

Synthetic degradable polyesters derived from monomers, such as lactide, glycolide and caprolactone have been widely used as sutures and bone fixation devices for 30 years (Griffith 2000). Lactic acid is a chiral molecule present in L and D isomers. The L-isomer is a biological metabolite. All polymers in this group are not soluble in water, but degrade by hydrolysis of their ester bond. Their mechanical and degradation properties are influenced by their crystallinity, the molecular weight (Mw), the glass temperature, and monomer hydrophobicity (Griffith 2000). Homopolymers can crystallize, but co-polymers are usually

amorphous. The rate of degradation is preferably caused by access of water to the ester bond (Schmitt *et al*. 1993). Water access to the ester bond is affected by the crystallinity of the sample, the hydrophobicity of the monomers (Schmitt *et al*. 1994), and the bulk sample dimensions (Grizzi *et al.* 1995). 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).

Among the homopolymers, PLA has been widely used in applications in which relatively slow degradation is required. Final degradation of PLA can take place after months or years. The local complications after long-term degradation periods may be due to the presence of crystalline degradation products (Böstman *et al.* 2000). A study by De Jong and co-workers (De Jong *et al.* 2005) evaluated a local reaction to polyethylene (PE), non-degraded PLA and pre-degraded PLA. At 2, 13 and 26 weeks after implantation, both PE and non-degraded PLA evoked the formation of a minimal layer of macrophage-like cells, and a small number of multinucleated giant cells at the implant interface with a mild capsule. Polymorphonuclear granular leukocytes (PMNs) were observed in week 2 in all three groups of implants. However, for pre-degraded PLA, PMNs were also regularly present in week 26 postoperatively. Moreover, pre-degraded PLA was almost degraded into small fragments at all time points; some of the fragments were phagocytized by multinucleated cells. In week 2, the infiltrate contained both macrophages and lymphocytes. Around the bigger parts of the polymer a fibrotic capsule was found. In week 13, large swollen macrophages with birefringent materials were present. In week 26 after implantation, polymer and cellular debris was present, probably released from dead macrophages. In all three groups, CD4 positive cells and a lesser degree of CD8 positive cells, indicating T helper and T suppressor/cytotoxic cells, were associated with the implant.

Polycaprolactone is another polymer with a relatively long degradation time that has been extensively studied in tissue engineering. The degradation of this homopolymer is of the order of two to three years, which is a much slower rate than that of PLA. In addition, it is compatible with other polymers (Gunatillake and Adhikari 2003). Poly ε-caprolactone based matrices supported adhesion, proliferation and calcium deposition of osteoblast-like Saos-2 cells during 1 month of culture. Calcium deposition of Saos-2 was further enhanced if hydroxyapatite micro-particles were embedded into the polymer (Ciapetti *et al*. 2003). To increase the hydrophilicity of polycaprolactone, it can be treated with sodium hydroxide. Processed polycaprolactone scaffolds were used for a co-culture of bone-marrow stromal cells

and chondrocytes, and enabled the formation of mineralized matrix throughout the scaffold (Cao *et al.* 2003).

On the other hand, in polyglycolides, the degradation time is relatively short, which can provoke a massive release of acid metabolites, followed by inflammation reactions. In patients treated with polyglycolide devices (e.g., screws for internal fixation of the bone), the first signs of an inflammatory foreign tissue reaction appeared 10-12 weeks after the operation. The typical reaction started with a suddenly emerging painful, erythematous, fluctuating papule over the implant track, which usually burst within a few days and revealed a sinus discharging liquid remnants of the disintegrated implant. The discharge persists from 6 weeks up to 6 months. In one half of the patients, the symptoms were accompanied by osteolysis along the implant tracks (Böstman and Pihlajamäki 2000). The biopsy showed a non-specific inflammatory foreign body reaction with abundant polymeric particles birefringent under polarized light that were surrounded by mononuclear phagocytes and multinucleated foreign-body giant cells (Böstman and Pihlajamäki 2000). In another study, PGA pins, implanted in a cancellous rabbit femur bone, did not show any acute inflammatory reaction, although the macrophages were present up to 12 weeks (Pihlajamäki *et al*. 2006).

Because of its rapid degradation, PGA is used mainly in copolymers with slowly degradable polymers, particularly PLA. An increased content of glycolic acid in PLGA (50:50 compared to 75:25) led to rapid degradation; the copolymer became more brittle and stiffer (Lu *et al*. 1999). PLGA degrades by simple hydrolysis of ester bonds into lactic and glycolic acid. All PLGA films degrade by heterogeneous bulk degradation. Interestingly, in PLGA foils, the surface layer degraded more slowly than the rapidly-degrading central part. Similarly, thick layers degraded faster than thin layers. This feature is caused by an autocatalytic effect (Lu *et al*. 1999). During the process of autocatalysis, the intermediate degradation products containing carboxylic groups were trapped inside the material until their molecular weight decreased to a critical value of 1,000 and became soluble in water (Park 1995; Lu *et al*. 1999). In the thicker PLGA samples (50:50, about 100 μm in thickness) macropores 10-40 μm in diameter formed at the surface in a late stage of degradation (Lu *et al*. 1999).

In addition, during the degradation process, the molecular weight of all PLGA decreased immediately after being placed in a PBS buffer and continued to decrease throughout the time course. On the other hand, the specific weight (i.e., the weight per volume unit) of PLGA films remained constant for several weeks, and then dropped dramatically. The time course of

strong weight loss corresponded to a rapid decrease in pH due to the release of acidic polymer degradation products.

In the form of three-dimensional foams, PLA were able to support proliferation and differentiation of both human fetal and adult osteoblasts (Montjovent *et al*. 2005). This osteogenic differentiaton was manifested by mineralization of extracellular matrix in the form of calcium phosphate crystals. In addition, this differentiation can be induced by combining degradable polymers with a mineral component. PLGA composites containing hydroxyapatite or bioactive glasses are bioactive, that is, they interact with the biological environment and can chemically integrate with the surrounding bone tissue *in vivo*. As a result, a layer of calcium phosphate is formed, through which the implant is chemically fixed to the surrounding bone tissue (Lu *et al*. 2003; Rezania and Healy 1999). In addition to their bioactivity, composites of PLGA and bioactive glass have other useful properties, such as improved compressive modulus similar to that of trabecular bone, increased cell adhesion, proliferation, collagen type I synthesis and higher cell differentiation. Degradation products of bioglass are alkaline and can buffer acidic pH during PLGA degradation. In the study, the pH in the cell cultures remained within physiological ranges during a 3-week period (Lu *et al*. 2003).

Implants made of polydioxanone and polyglycolide-co-trimethylene-carbonate have been used less frequently in medicine, but sporadic foreign-body reactions have been reported for these materials (Böstman and Pihlajamäki 2000). A polydioxanone screw implanted in a cancellous rabbit bone did not cause any macroscopically manifest signs of inflammation, although a short-time presence of macrophages around the implant was observed. On the other hand, polydioxanon slightly decreased the activity of osteoblasts compared to both PGA and PLLA (Pihlajamäki *et al*. 2006).

Vascular grafts prepared from a co-polymer of PGA and polyhydroxyalkanoate remained patent for 5 months after implantation into lamb abdominal aortas only if they had been seeded with a mixture of autologous smooth muscle cells, endothelial cells and fibroblasts; the acellular controls were occluded due to thrombosis (Shum-Tim *et al*. 1999; Baguneid *et al*. 2006)

In addition, degradable polymers have been combined with non-degradable polymers in order to improve the mechanical properties and the durability of the scaffods for sufficient tissue regeneration. For example, partially resorbable bioarteficial vascular prostheses, succesfully tested in dogs, were woven from composite yarns containing 69% polyglactin 910

and 31% polypropylene, or containing 70% polydioxanone and 30% polypropylene (Greisler *et al.* 1991).

1.3. Proteins used for tissue engineering

1.3.1. Fibrin assemblies

Fibrinogen and fibrin play an important role in blood clotting, fibrinolysis, cellular and matrix interaction, inflammation, wound healing and neoplasia (Mosesson *et al*. 2001). In addition, fibrin is known as a biocompatible and biodegradable material interacting with tissue without inducing inflammation and thrombosis, foreign body reaction, tissue necrosis or extensive fibrosis (Bense and Woodhouse 1999; Kannan *et al*. 2005; Jackson 2001; Hasegawa 2005).

1.3.1.1. Fibrin in wound healing and inflammation

In the last step of the coagulation cascade, thrombin cleaves fibrinogen, and a fibrin clot is formed. The fibrin clot is subsequently stabilized by the cross-linking activity of coagulation factor XIII (Mosesson *et al*. 2001). Thrombin has a catalytic protease centre and two positively charged patches located at opposite ends of the molecule called anion-binding exosites I and II (DiCera 2003). During the formation of a fibrin gel, thrombin can be bound to fibrin in two orientations. In the former, thrombin is bound via both the fibrin recognizing exosites and the catalytic centre. In the latter, thrombin is bound only via exosites while the thrombin catalytic centre remains accessible for fibrinogen cleavage, i.e. the thrombin remains active. Unlike thrombin circulating in blood, this bound thrombin is protected against inactivation by the heparin-antithrombin III treatment commonly used in medical practice; however, it can be inactivated by antithrombin III-independent inhibitors, such as hirudin and D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Rubens *et al*. 1993; Laurens *et al*. 2006). These inhibitors can be used to prepare thin fibrin assemblies that cover the surface of large pores inside the synthetic scaffolds without filling them up with bulk fibrin gel to provide space for cell migration and the exchange of nutrients and waste.

Fibrin stability is maintained by crosslinking with transglutaminase, factor XIII or XIIIa, which catalyses the formation of γ dimers. EC were observed to proliferate and migrate on fibrin gels coated with factor XIIIa, but not with factor XIII (Dallabrida *et al*. 2000). Cross-

linked γ chains in fibrin are located between the D domains of opposing strands of cross-linked fibrin fibrils. This arrangement can explain the superior viscoelastic properties of fibrin. After a maximum stretch of up to 1.8 times its length, only cross-linked fibrin can recover its original form (Mosesson 2005). Apart from the γ chain, the α chain takes part in crosslinking, but this reaction is slower. A complete α and γ chain cross-linked fibrin gel is essential for adhesion and migration of fibroblasts and macrophages. During cross-linking, fibronectin is covalently bound to fibrin, providing more specific sites for migrating cells (Grinnell *et al.* 1980).

Specific sites of fibrinogen and fibrin interact with proenzymes, enzyme inhibitors, clotting factors, cell receptors and growth factors, such as FGF-2 and IGF-1; IGF-1 is bound to insulin-like growth factor-binding protein-3 (Mosesson *et al*. 2001). The fibrin β15-42 sequence is able to bind heparin, thereby participating in cell-matrix interactions. The same sequence mediates platelet spreading, EC spreading, proliferation and capillary formation, release of von Willebrand factor, and fibroblast proliferation (Mosesson 2005).

Fibrinogen contains two binding sites for integrin adhesion receptors on cells. These sites contain a specific amino acid sequence RGD that binds $\alpha_{IIB}\beta_3$ integrin of platelets and $\alpha_v\beta_3$ integrin of EC, melanoma cells and fibroblasts (Mosesson *et al*. 2001). Both integrin receptors $\alpha_3\beta_1$ (which binds to fibronectin, laminin-5, or entactin), and $\alpha_5\beta_1$ were upregulated on periwound fibroblasts the day prior to moving them into the wound clot (Xu and Clark 1996; Clark 2001). $\alpha_v\beta_3$ integrin binds vitronectin (through RGD), fibronectin, fibrinogen, fibrin, von Willebrand factor, and thrombospondin (Clark 2001). $\alpha_v\beta_3$ integrin promotes EC migration on provisional matrix proteins. Fibrin, but not collagen gels, induced $\alpha_v\beta_3$ expression on cultured human microvascular EC (Feng *et al*. 1999; Clark 2001). Moreover, in porcine cutaneous wounds $\alpha_v\beta_3$ integrin expression was observed on the tips of the capillary sprouts as they invaded the fibrin clot, and also in hypertrophied vessels adjacent to the wound during invasive angiogenesis or granulation tissue formation (Clark *et al.* 1996). In contrast, mature blood vessels showed little or no staining for $\alpha_v\beta_3$ integrin. In both EC and fibroblasts, early wound (also called provisional) matrix proteins therefore stimulated the expression of provisional matrix integrins. Monocytes and neutrophils bind to fibrinogen through $\alpha_M\beta_2$ receptors (Mosesson *et al*. 2001).

A fibrin clot provides a temporary scaffold for the influx of fibroblasts, EC, monocytes as well as VSMC (Clark 2001; Sahni and Francis 2000). Fibroblasts, presumably stimulated by PDGF and TGF-β, fibrin and fibronectin, proliferate, express appropriate integrin receptors, and migrate into the wound space. Fibroblasts synthesise the ECM necessary for cell

ingrowth. The provisional matrix contains fibrin, fibronectin and vitronectin, and is later composed of hyaluronan and fibronectin produced *in situ*. Fibronectin serves as a conduit for cell migration, hyaluronic acid supports cell invasion, and fibrinogen is a reservoir of cytokines (Clark *et al*. 1982; Clark 2001).

Angiogenesis, the process of new blood vessel formation, depends on appropriate wound bed and EC alteration, stimulated migration and mitogenic stimulation of EC (Clark 2001). The following soluble factors participate in angiogenesis: bFGF, acidic FGF, VEGF, angiopoietin, PDGF, TGF-α, TGF-β, and others (Clark 2001; Folkman and Klagsbrun 1987). To induce angiogenesis, fibrin must provide EC with a three-dimensional matrix that supports cell migration, and must express a chemotactic activity that is sufficient to stimulate EC migration into the fibrin clot. On purified human fibrin *in vitro,* no angiogenesis was observed. However, angiogenesis was stimulated in a dose-dependent manner, if VEGF or bFGF were added (Clark 2001). In addition, the tube formation by bovine EC in response to bFGF or VEGF was increased only in malleable fibrin gel polymerised at a pH of 7.4-7.6, but did not change in rigid, opaque fibrin gels polymerised at pH 6.8-7.2. On the other hand, the migration of EC in fibrin gels was inversely proportional to the capillary morphogenesis (Nehls and Herrmann 2006).

The VEGF family, which comprises six related proteins: VEGF A-E and placenta growth factor, are important stimulating factors for angiogenesis. They stimulate cells to produce matrix MMPs that degrade the basement membrane and the surrounding ECM. This results in EC proliferation and migration towards the interstitium, where they sprout (Davis *et al.* 2002; Nillesen *et al.* 2007). Subsequently, pericytes start their proliferation and migration, and form a single cell layer around the sprout. Another growth factor involved in angiogenesis is FGF-2. FGF-2 stimulates endothelial cells to synthesise both MMPs and VEGF, and increases the expression of VEGF receptors. FGF-2 also modulates EC proliferation, migration, protease production, and expression of cadherin and integrin receptors. In addition, FGF-2 upregulates gap junctions among EC, and increases pericyte attraction and matrix deposition (Presta *et al*. 2005).

In addition to its role in angiogenesis, VEGF is also necessary for bone formation. VEGF regulates the recruitment, survival and proliferation, migration and differentiation of osteoblasts and osteoclasts in an autocrine manner (Hsiong and Mooney 2006). In addition, VEGF directly stimulates EC production of bone-forming factors for osteoblasts. VEGF expression in osteoblasts is upregulated by many osteoinductive factors, such as TGF-β1, FGF-2 or IGF-1 (Hsiong and Mooney 2006).

Both monocytes and neutrophils are attracted to the site of an injury by fibrinopeptides cleaved from fibrinogen by thrombin, and from fibrin by plasmin (Clark 2001). Monocytes adhered to ECM molecules are stimulated to undergo metamorphosis into either inflammatory or reparative macrophages. Their adherence induces the mRNa expression of colony stimulating factor-1 that is necessary for monocyte/macrophage survival; tumor necrosis factor-α, a potent inflammatory cytokine; PDGF, a potent chemoattractant and mitogen for fibroblasts; and transcription-associated proteins c-fos, c-jun, and IκB (Juliano and Haskill 1993; Clark 2001).

Activated neutrophils release proteases that facilitate their penetration as well as fibrin clot degradation. In addition, they destroy bacteria at the wound site via phagocytosis associated with the production of toxic oxygen radicals.

Fibrin seems to accumulate the newly synthesised collagen and other ECM components (Jockenhoevel *et al*. 2001). *In vitro* experiments confirmed that fibrin promotes matrix synthesis through the release of PDGF and TGF-β (Jockenhoevel *et al*. 2001). Fibrin is degraded by proteolytic action of plasmin and matrix metalloproteinases, and is gradually replaced by collagen and other ECM compounds produced by cells (Clark 2001; Bense and Woodhouse 1998).

1.3.1.2. Fibrin assemblies for cell seeding

Blombäck and co-workers have shown that the architecture of fibrin prepared from low levels of thrombin is more branched and less porous than networks from high concentrations of thrombin. Fibrin is branched through so-called trimolecular or equilateral junctions of fibrin fibrils that enhance fibrin clot elasticity. Junctions of another kind, referred to as tetramolecular or bilateral, confer strength and rigidity to the network fibers (Blombäck *et al*. 1994; Mosesson *et al*. 2001).

The rigidity of the fibrin was critical for fibrin matrices with human mesenchymal stem cells. The human mesenchymal stem cells were more viable if the thrombin activity was 50 IU/ml or 100 IU/ml. A further increase in thrombin concentration decreased the viability of the cells (Bensaid *et al.* 2003). In addition, in the most rigid fibrin glue with thrombin concentration higher than that of 250 IU/ml, cells failed to spread or proliferated and appeared rounded after 4-5 days in culture, and no fibrin clot lysis was observed. The optimum rigidity of the fibrin (100 IU thrombin) allowed manipulation after 15 days; concurrently, the fibrin showed limited fibrin degradation.

Fibrin sealant applied to skin grafts increased the rate of granular tissue formation and promoted both neovascularisation and reepithelialisation (Bense and Woodhouse 1999). Fibrin sealant, also referred to as "fibrin glue", aids hemostasis and atraumatic tissue union during surgery. Fibrin sealant applied to a sutured skin graft reduced wound contraction compared to the control with normal saline (Brown *et al*. 1992; Jackson 2001).

Fibrin glue provides an underlying protein on the inner surface of synthetic polymeric vascular grafts and may significantly accelerate their endothelialisation and reduce intimal hyperplasia. For vascular prostheses the thickness of the fibrin layer is in the range from 4 to 800 μm (Fernandez *et al*. 2005). Bone marrow cells, cells from venous or adipose tissues can be used to improve healing and reduce anastomotic intimal hyperplasia (Matsumoto *et al*. 1995; Cardon *et al*. 2000; Walpoth and Bowlin 2005). Fibrin can be advantageously produced from the patient's blood as an autologous scaffold without the risk of foreign body reaction. Thrombin-free fibrin-coated small-diameter vascular prostheses in rabbit had superior anti-thrombogenicity compared to conventional fibrin-coated prostheses (Hasegawa *et al.* 2005).

The disadvantages of fibrin comprise its poor mechanical properties, relatively quick degradation, and shrinkage due to structural changes and due to contraction of the newly synthesised collagen bundles (Jockenhoevel *et al*. 2001). Fibrin degradation and remodelling are supported by cell-associated enzymatic activity, and can be slowed down by adding aprotinin, an inhibitor of plasmin, or ε- aminocaproic acid, an inhibitor of fibrinolysis, into the cultivation medium (Grassl *et al*. 2003). The shrinkage can be reduced by chemical or mechanical fixation of the gel. Gel fixation, on the one hand, and shrinkage forces, on the other, can lead to mechanical stress in the tissue, which may induce collagen synthesis and in turn improve the mechanical properties of the scaffold (Jockenhoevel *et al*. 2001). In addition, collagen synthesis of neonatal VSMC embedded in fibrin gel increased threefold after supplementation with TGF-β and sixfold with both TGF-β and insulin. TGF-β probably influenced collagen synthesis by the increase in procollagen mRNA and by stimulating the synthesis of the enzyme lysyl oxidase that crosslinks collagen. Insulin is known to increase the residence time of procollagen mRNA and to upregulate latent TGF-β production. The collagen newly synthesised by VSMC greatly improved both the strength and the stiffness of the fibrin scaffolds. Much lower improvement of the mechanical properties of the gel was observed when neonatal VSMC were embedded in collagen gel or when fibroblasts were embedded in fibrin gel with the same supplementation (Grassl *et al*. 2003).

A technique of *in situ* polymerization of fibrin with cell suspension assures homogeneous distribution of the cells inside the scaffolds. Encapsulation of the cells inside

the fibrin gel was used for cartilage regeneration, molding a valve conduit containing myofibroblast cells (Van Susante *et al.* 1999; Jockenhoevel *et al*. 2001), bone grafts containing human mesenchymal stem cells (Catelas *et al.* 2006), or fibrin was used as a matrix for orthotopic extravascular transplantation of hepatocytes (Bruns *et al.* 2005).

For some cell types, encapsulation of the cells inside the gel is not an optimal approach. Olfactory ensheathing glial cells seeded inside the fibrin gel proliferated and formed tentacles, while NE-4C neural stem cells degraded the surrounding fibrin gel and grew into clusters floating inside the formed cavities and finally died. When seeded on the surface of a fibrin gel, mesenchymal stromal cells grew in a thin layer on the surface without migrating into the bulk (Riedel *et al*. 2004).

1.3.2. Proteins of extracellular matrix in tissue engineering

Extracellular matrix is a natural scaffold that provides cells with mechanical support as well as a microenvironment to which they can respond. The preparation of artificial matrix or scaffolds that mimic natural ECM is a common approach in tissue engineering (Duan *et al*. 2007).

Type I collagen is an important component of the vessel wall, and a major matrix protein of bone and other connective tissues (Tye *et al*. 2005), and is advantageously used for tissue engineering of both vessels and bone. A vascular tubular hybrid collagenous scaffold was prepared by pouring a cold mixed solution of canine jugular smooth muscle cells and type I collagen into a tubular mould. After thermal gelatinization and 10-day cultivation *in vitro*, the tube was seeded with canine jugular endothelial cells (Hirai *et al.* 1994; Baguneid *et al*. 2006). The collagen concentration was able to control the rupture pressure of the conduit up to 110 mm Hg.

In another study, collagenous and gelatine grafts releasing bone sialoprotein were implanted into a critical-sized 8-mm calvarial defect that does not heal spontaneously. Bone sialoprotein was reported to stimulate cell attachment and proliferation of fibroblasts, osteoblasts and osteoclasts, increased calcification of newly synthesized organic matrix, expression of an osteoblastic phenotype in experiments with MC3T3-E1 preosteoblast cells, bone derived osteoblasts, and bone marrow cells (Mizuno *et al.* 2000; Xu *et al*. 2007). BSP is known to interact with reconstituted fibrillar collagen in the “hole zones” of type I collagen fibrils, a site associated with early mineralization (Fujisawa and Kuboki 1992). Collagen scaffolds were composed of fibrils/fibres, but gelatine lacked the primary fibrous structure.

Both scaffolds induced cell proliferation in the *dura mater* and the adjacent areas, but only collagen-BSP implants induced early mineralization and enhanced the formation of new bone matrix after 30 days (Xu *et al.* 2007).

Collagen-heparin scaffolds releasing either FGF-2 or VEGF, or both growth factors, were subcutaneously implanted in rats to observe both angiogenesis and vessel maturation (Nillesen *et al*. 2007). On day 7, vessels were observed in collagen scaffolds releasing growth factors, but not in the pure collagen. The collagen scaffold releasing both VEGF and FGF-2 contained the largest blood vessel area, as well as the highest percentage of matured vessels, measured as vessels positively stained for smooth α-actin. In addition, the same scaffold contained almost no hypoxic cells on day 7. Similarly, collagen-heparan sulphate matrices releasing bFGF, implanted in rats, triggered angiogenesis after a week. This means a significant reduction in the time needed to induce angiogenesis in the graft, from the usual 3-4 weeks to one week (Pieper *et al*. 2002).

In VSMC cultured in the classical "two dimensional" cell culture system on collagen I or fibronectin layers deposited on culture dishes, both collagen type I and fibronectin induced a shift of VSMC towards the synthetic state, characterized by a loss of contractile filaments and inceased proliferation activity (Yamamoto *et al*. 1993; Stegemann *et al*. 2005). Decreasing proliferation activity (e.g. by reaching confluence or cultivation in defined serum-free media) is usually associated with VSMC differentiation towards the contractile phenotype, manifested by increased smooth muscle α-actin expression. On the other hand, three-dimensional collagen type I gel greatly reduced both VSMC proliferation and smooth muscle α-actin expression compared to two-dimensional collagen. These observations demonstrate the noticeably different behaviour of VSMC on two- and three-dimensional ECM matrices (Stegemann and Nerem 2003). Biochemical stimulation of VSMC embedded in a three-dimensional collagen gel by TGF and insulin increased their production of collagen and improved the mechanical properties of the gel (Grassl *et al*. 2003; Stegemann *et al*. 2005).

Fibronectin is an extracellular matrix protein that promotes cell attachment and spreading (Potts and Campbell 1996; Calonder *et al*. 2005). The specific amino acid sequence RGD responsible for cell binding through integrin receptors ($\alpha_5\beta_1$) is located on the 10^{th} type III repeat module, and synergy sites PHSRN are on the 8^{th} and 9^{th} type III repeats of fibronectin. Upon immobilisation, fibronectin exhibits an increased beta sheet conformation. In addition, novel cell binding sites in fibronectin are exposed on the surface compared to a soluble form (Aota *et al*. 1994; Iuliano *et al*. 1993). The ordinary immobilisation procedure results in a random distribution of protein orientations on the surface, leaving a fraction of the binding

sites inaccessible to cells. Oriented placement of fibronectin was achieved by preplacing a monoclonal antibody targeted to a region of fibronectin away from the cell binding sites. This procedure led to increased density and thickness of the adsorbed fibronectin layer (Calonder *et al*. 2005). There was greater and faster spreading of HUVEC, but their elongation was slower on oriented fibronectin layers compared to the random orientation.

The orientation of fibronectin molecules adsorbed to a material surface was markedly influenced by the physicochemical properties of this surface, particularly its wettability. On moderately hydrophilic surfaces, increased accessibility of the cell-binding domain in the fibronectin molecule to cell adhesion receptors was observed. As a result, the cells spread better and formed more focal contacts on these surfaces. Cell detachment upon stress was lower on hydrophilic glass compared to hydrophobic silane at low concentrations of fibronectin. At higher concentrations, the cell detachment was equal on both surfaces. Increased fibronectin concentration prevented cell detachment following exposure to 60 dynes/cm^2 on both surfaces (Iuliano *et al.* 1993). Fibronectin coating of polytetrafluoroethylene (PTFE) prostheses enhanced their endothelialisation regardless of whether they were seeded with EC (Seeger and Klingman 1988). Co-adsorbed albumin improved poor cell spreading on fibronectin pre-adsorbed on hydrophobic surfaces (Horbett 1993). A possible explanation is that the co-adsorption of albumin and fibronectin influences fibronectin conformation. This phenomenon, called "albumin rescuing", caused higher availability of fibronectin cell binding domains on surfaces such as tissue culture polystyrene, microbiological polystyrene, and Teflon AF® (Koenig *et al*. 2002) if low concentrations of albumin were used. Endothelial cells attached to these surfaces in the same density (in a standard serum-supplemented medium) or in a lower density (in a protein-free medium) compared to pure fibronectin. Although the most hydrophobic Teflon AF® exhibited reduced EC attachment and spreading, the expression of both RhoA and endogenous fibronectin were up-regulated. RhoA, a small GTPase, regulates and promotes cell spreading and adhesion to substrates; its concentration is reduced when cells enter a quiescent state after reaching confluent levels. Thus, endogenous fibronectin helps the cells to survive on the inconvenient hydrophobic Teflon surface, as fibronectin possesses binding sites that permit its self association (Iuliano *et al.* 1993; Koenig *et al*. 2002).

Laminin, a complex trimer glycoprotein, is a major component of the basement membrane present in 15 functional isoforms. Laminin is a component of a basement membrane together with collagen IV isoform, nidogens, a few proteoglycans (agrin, perlecan), fibulins, and a number of part-time associated components, including type XV

collagen and type XVIII collagen. Basement membranes concentrate and present growth factors via glycosaminoglycans in proteoglycans (Ekblom *et al*. 2003). Cell integrin receptors (e.g. $\alpha_6\beta_1$, $\alpha_6\beta_4$, and $\alpha_7\beta_1$) interact with specific amino acid sequences in laminin that include RGD and IKVAV sequences of the α-chain, YIGSR of the β_1-chain, and RNIAEIIKDI of the γ-chain. Other motifs of laminin bind to dystroglycan, cell surface sulfatides, and heparin (Meiners and Mercado 2007; Duan *et al*. 2007; Ekblom *et al*. 2003).

Primary airway smooth muscle cells cultivated on laminin slowed down their proliferation; they had a lower response to mitogens, and expressed more contractile proteins in comparison with cells cultivated on type I collagen or plastic. This suggests that laminin maintains the contractile phenotype of airway smooth muscle cells, and prevents phenotype modulation to the synthetic/proliferative type (Hirst *et al*. 2000). A similar effect was observed on visceral and vascular smooth muscle cells (Tran *et al*. 2006; Ekblom *et al*. 2003). As revealed in a study with soluble laminin mimetic peptides (YIGSR and GRGDSP), endogenously expressed laminin is both required and sufficient to promote phenotypic maturation of airway smooth muscle cells (Tran *et al*. 2006), however, exogenous laminin lacks this ability. Soluble laminin mimetic peptides (YIGSR and GRGDSP) added into the cultivation medium competitively inhibited the binding of endogenously expressed laminin. This in turn abolished accumulation of contractile phenotype markers, such as desmin and calponin, although cell morphology, viability, and proliferation were not affected.

Tissue culture plates coated with fibronectin, type I and IV collagens, or vitronectin enhanced 6- to 8-fold the adhesion of human mesenchymal stem cells compared to an uncoated culture dish and laminin. Interestingly, osteogenic differentiation of mesenchymal stem cells, proved by increased expressions of type I collagen, osteopontin, and alkaline phosphatase, was supported on both vitronectin and type I collagen if cultivated in either a DMEM medium or an osteogenic medium (Salasznyk *et al*. 2004). These results demonstrate the influence of ECM alone on cell behaviour.

Interestingly, the entire cell sheets with their ECM were used to produce tissue-engineered blood vessels. An acellular inner membrane was formed from dehydrated tubular fibroblast sheet, and it was slipped around a perforated tubular PTFE mandrel. A sheet of VSMC, rolled around it, produced a vascular medium; a sheet of fibroblasts provided adventitia. After removing the mandrel, EC were seeded at the lumen. This entire biological conduit had the characteristics of a natural vessel and was strong enough to withstand physiological blood pressure without additional reinforcement (L'Heureux *et al*. 1998).

1.3.3. Other proteins in tissue engineering of vessels and bone

Silk fibroin is a natural protein derived from raw silk, which slowly degrades in the body. It contains (Ala-Gly)n and (Ala)n repeated aminoacids; the nonpolar character of these aminoacids reduces its hydrophylicity (Inuvye *et al.* 1998; Cai *et al*. 2002). Its properties, such as low thrombogenicity, non-toxicity and low-immunogenicity, ability to be easily modified, and good mechanical properties make it a promising material for tissue engineered vessels (Baguneid *et al*. 2006; Lovett *et al*. 2007). In cultures with human colon adenocarcinoma cells, both silk fibroin- and collagen-coated polysterene improved cell attachment and growth to a similar extent compared to pure polystyrene. On the other hand, the growth of insect cells was not affected by silk coating (Inouye *et al*. 1998). In another study, surface modification of PDLLA with silk fibroin improved the adhesion, proliferation and differentiation of osteoblasts (Cai *et al.* 2002).

Silk fibroin in blends with PEO was used to prepare microvascular grafts with an inner diameter of 1 to 6 mm (Lovett *et al.* 2007). Increased concentration of PEO in a blend up to 20 % increased the average pore size in the graft wall, decreased burst pressures, and had a barrier function for both protein diffusion and cell migration. The burst pressures of silk microtubes without pores or with small pores (from 100/0 to 98/2 wt % silk fibroin/PEO) were higher than those of human saphenous vein (Lovett e*t al*. 2007). The optimal composition contained 98/2 wt % silk fibroin/PEO; it had sufficient permeability to small proteins and limited the migration of EC (Jin and Kaplan 2003).

Pseudo-poly(amino acids) are polymers based on natural aminoacids linked by nonamid bonds (Griffith 2000). They have improved stability, mechanical properties and processing, and are easy to prepare. In this group, poly(tyrosine carbonates) seem to be promising for orthopaedic applications.

1.4. Nanostructured surfaces

Nanostructured and nanofibrous materials mimic the geometrical conformation and topography of ECM proteins and also inorganic ECM components. For example, lamellar bone contains inorganic mineral grains 20 nm in length and 2-5 nm in diameter. Nanostructured surfaces are able to alter the adsorption of cell adhesion-mediating proteins, i.e., their geometrical conformation and exposure or hiding of cell-binding sites of the proteins, and thus they influence cell adhesion and behaviour (Lord *et al.* 2006).

Matrix replicas prepared from denuded vessels revealed a highly complex geometry at cellular and subcellular levels (Goodman *et al*. 1996). On these textured replicas made of polyurethane, more rapid initial spreading of EC was observed. However, in a confluent layer, EC were more rounded on the textured surface in comparison with smooth polyurethane.

EC cultured on nanostructured silk fibroin nets coated with fibronectin up-regulated β_1-integrin gene expression compared to EC on a microstructured surface. Since β_1-integrin is commonly involved in cell adhesion to collagen, cells seemed to recognize nanostructured silk fibroin like a natural environment. In addition, EC developed focal adhesions containing focal adhesion kinase, vinculin and phosphocaveolin-1, which were aligned along the fibres. The distribution of phosphorylated and non-phosphorylated caveolin-1 was characteristic for polarised and migrating cells (Bondar *et al*. 2008). In another experiment, a decreased number of spread EC was observed on nanorough poly(4-bromostyrene), compared to smooth surfaces (Barbucci *et al*. 2003). The finest nanoroughness supported EC proliferation and spread morphology with pseudopodia, which adhered to the top of the nanohills.

Nanorough PLGA scaffolds increased the adhesion and growth of bladder smooth muscle cells compared to a sub-micron structured scaffold. Moreover, a higher elastin content was observed on nanostructured PLGA compared to a micro-dimensional surface. On the other hand, collagen synthesis was the same, regardless of surface roughness (Pattison *et al*. 2005).

Mesenchymal stem cells seeded on non-woven nanofibre poly(ε-caprolactone) created multilayers after four-week cultivation; in addition, osteoblast-like cells and abundant calcification were found there (Yoshimoto *et al*. 2003). Nanostructured surfaces composed of silicon nanowires of different density and length supported osteoblast adhesion, proliferation and alkaline phosphatase production. The maximum effect on cell growth and differentiation was observed on a dense net with long wires (Popat *et al*. 2006).

On nanostructured surfaces, preferential adhesion of osteoblasts over other cell types, mainly fibroblasts, has been reported (Webster *et al*. 2000; Price *et al.* 2004). The underlying mechanism seems to be preferential adsorption of vitronectin on these surfaces, which is due to its relatively small and less complicated molecule in comparison with other ECM proteins. Therefore, in bone tissue engineering, nanostructured surfaces could prevent the fibrous encapsulation of the bone implant.

1.5. Anti-adhesive materials

As mentioned above, cells do not adhere directly to a polymer surface, but their adhesion is mediated by proteins adsorbed from blood or from a cultivating medium. Biological fluids contain a variety of proteins, including those that mediate cell adhesion. The proteins adsorb strongly to the surfaces due to several forces. These include attractive and repulsive interactions, such as electrostatic interactions between the interface and the protein, Van der Waals interaction, long-range Coulomb interaction due to the net charge of the surface and the protein, and the dipole moment. Other processes that contribute to protein adsorption are dehydration of the interface, structural changes, e.g. unfolding in the protein molecule, or dipole-dipole interactions between the proteins on the surface (Duinhoven *et al*. 1995; Griffith 2000).

On the adsorbed proteins, the cells adhere through specific sites in these molecules, recognized by cell adhesion receptors on the cell membrane. These binding sites usually comprise specific amino acid sequences, such as RGD, and their cooperating sequences, e.g. PHSRN. The most known and systemized cell adhesion receptors are integrins, though non-integrin cell adhesion receptors have also been desribed. These receptors are often based on saccharide-containing molecules (e.g., proteoglycans) and bind oligopeptidic or saccharide-based ligands (for a review, see Bacakova *et al*. 2004; Balasundaram and Webster 2006).

After binding their ligands, the cell adhesion receptors are recruited into specific domains in the cell membrane, called “focal adhesion plaques”, “focal adhesion sites”, “focal adhesion contacts” or “focal adhesions”, where they communicate with a wide range of structural and signalling molecules, such as talin, vinculin, paxillin, alpha-actinin and focal adhesion kinase. Focal adhesion plaques are associated with the actin cytoskeleton, and further communicate with other molecules and cellular components, including the nucleus. In this way, the signal from the adhesion substrate, represented by an artificial material, can influence gene expression and further cell behaviour, e.g. the extent and strength of cell adhesion, proliferation activity, the switch between a proliferation and differentiation program, apoptosis, or synthesis of specific functional molecules (for a review, see Bacakova *et al*. 2004).

However, for the construction of bioinert implants, it is necessary to inhibit the protein adsorption in order to minimize the cell-implant interaction, e.g. adhesion of platelets and immunocompetent cells on the inner surface of conventional non-endothelized polymeric vascular prostheses. In addition, even if cell adhesion is desirable, spontaneous protein

adsorption on most materials conventionally used for the construction of body implants is less controllable. In advanced tissue engineering, it is necessary to functionalize the biomaterial surface with ligands for cell adhesion receptors in a defined amount, spectrum, concentration and spatial distribution. An appropriate background for the attachment of these ligands is represented by surfaces that do not allow spontaneous protein adsorption and cell adhesion (Bacakova *et al.* 2007).

Various approaches have been used to inhibit protein adsorption, such as a polyethylene oxide (PEO) brush, radiation grafting, formation of interpenetrating networks, and modification of the polymer ends with an oligomeric or polymeric segment that has a thermodynamic driving force to assemble at the interface (Griffith 2000).

Highly hydrophilic, uncharged hydrogels with antiadhesive properties, such as poly(ethylene glycol) diacrylate, polacrylamid, thermally reversible N-isopropylacrylamid, and agarose gel became early model systems presenting small bioactive ligands against an inert background (Hern and Hubbell 1998; Griffith 2000; von Recum *et al.* 1998).

A PEO brush is most commonly used to create a protein-resistant surface (Irvine *et al.* 2001; Wang *et al.* 2002; VandeVondele *et al.* 2003; Xu *et al*. 2004). The mechanism of PEO brush inhibition of protein adsorption is in part attributed to steric hindrance due to the self repulsion of PEO in water. Protein resistance generally increases with a longer chain and higher grafting densities (Griffith 2000). In addition, flexible chains of PEO can provide suitable linkers for the attachment of adhesion oligopeptides, which ensure their good accessibility for cell adhesion receptors (Bacakova *et al.* 2007).

Besides PEO, other natural or synthetic molecules have been used to create bioinert surfaces, e.g., albumin, hyaluronic acid (Matsuda *et al*. 1992), polyvinyl alcohol or dextran (Amanda and Mallapragada 2001; VandeVondele *et al.* 2003; Griffith 2000), and amorphous hydrogenated diamond-like carbon (for a review, see Grinevich *et al.* 2008). This strategy has been used for constructing intraocular lenses (Banerjee *et al*. 2000), surfaces of heads and cups of artificial joints (Bacakova *et al*. 2001; Grinevich *et al.* 2008, in press) or surfaces of blood-contacting devices (Han *et al*. 1993).

Other means for controlling both protein adsorption and cell adhesion include thermoresponsive poly(N-isopropyl acrylamid) brushes that can be reversibly switched from a low-temperature biologically inert state to a high-temperature protein and a cell adhesive state (Hatakeyama *et al*. 2006; Xu *et al*. 2004). In addition, they can be functionalized by covalent conjugation to biologically active groups, mainly cell-binding peptide motifs binding cells (VandeVondele *et al.* 2003).

1.6. Functionalized materials

Functionalized materials are biologically active interfaces prepared from biologically inert polymers functionalized by specific peptides or proteins, such as growth factors, to introduce biochemical stimuli (Tugulu *et al.* 2007).

Poly(2-hydroxyethyl methacrylate) and poly(ethylene glycol methacrylate) effectively prevent non-specific and uncontrollable cell adhesion. The same polymers functionalized with RGD-containing oligopeptide (GGGRGDS) induced adhesion, spreading and proliferation of human umbilical vein endothelial cells. The cells on RGD grafted surfaces had large mature focal adhesions located mainly at the cell periphery. On control polymers without RGD grafting, the cells attached in low densities, and remained round-shaped; they developed a relatively high number of small focal adhesions at the periphery as well as fibrous adhesions in the central regions of the cells. RGD-grafted poly(2-hydroxyethyl methacrylate) and (polyethylene glycol methacrylate) with a confluent layer of EC were subjected to a shear stress of 15 dynes/cm^2 for 24h. The cells elongated in response to shear stress, developed stress fibres oriented in parallel with the long axis of the cells and parallel to the direction of flow. This reaction of EC represents a physiological response to the applied shear stress (McCue *et al*. 2004).

Myocardial ECM contains a wide variety of proteins such as fibronectin, vitronectin, type I, II and V collagen and metalloproteases (MMPs), and plays an important role in ventricular remodelling after mechanical overload and hypertrophy. In an *in vitro* study, neonatal rat myocytes were seeded on silicone membranes with either RGD or YIGSR covalently attached in different concentrations as well as with their respective peptides, fibronectin and laminin adsorbed noncovalently (Boateng *et al*. 2005). An increased concentration of both RGD and YIGSR enhanced myocyte attachment; however, this attachment did not reach the level found on the entire fibronectin molecules. The striated myocyte morphology was observed on fibronectin or laminin, but fewer muscle striations were visible on both oligopeptides. Moreover, a focal adhesion kinase was reduced by 50% in myocytes on YIGSR compared to the cells on laminin. In the myocardium, connexin plays an important role in cell-cell communication through gap junctions. Cardiac myocytes on both RGD and YIGSR peptides contained a significantly higher ratio of a phosphorylated form of connexin-43, which is typical for adult atrial cell phenotype. In myocytes on laminin, both phosphorylated and dephosphorylated forms of connexin-43 were found, a characteristic sign of both atrial and ventricular forms of myocytes. In addition, the increased connexin phosphorylation suggests

that the cells tried to compensate the weakened cell-substrate by enhancing the cell-cell communication. Similarly, in mesenchymal stem cells in cultures on surfaces patterned with hydrophilic and hydrophobic domains, the lower cell adhesion to the hydrophobic domains was partly compensated by an increased concentration of CD 44, i.e. a hyaluronan receptor participating in cell-cell adhesion (Filova *et al.* 2008b). On the other hand, the adhesion oligopeptides prevented myocyte detachment on mechanical stimulation compared to the adsorbed laminin; on YIGSR, this was accompanied by a reduction in expression of the myosin heavy chain (Boateng *et al*. 2005).

The use of cell binding through RGD-containing oligopeptides only, i.e. without an synergy site represented by PHSRN, decreased the cellular responses due to a less appropriate spatial conformation of the ligand, and thus altered the integrin signalling. As an alternative to short synthetic oligopeptides, fragments of ECM proteins can be used, such as recombinant fibronectin fragments containing both the RGD and PHSRN sequences needed for $\alpha_5\beta_1$ integrin binding (Cutler and Garcia 2003), the bone and cartilage stimulating peptide (BCSP^{TM}-1), or a synthetic fragment of human type I collagen that was observed to promote calvarial cells differentiation (Wang *et al*. 2008).

Both the adhesion and migration of microvascular EC or human VSMC were tested on PEG grafted with YIGSR, RGD, PHSRN, RGD/PHSRN and RGD/YIGSR (Fittkau *et al*. 2005). On RGD-functionalized PEG, the maximum adhesion of both cell types was achieved at an RGD concentration of 0.32 pmol/cm^2. The VSMC increased their adhesion to RGD/YIGSR compared to RGD/PHSRN and RGD, but their migration was highest on RGD-grafted PEG. Although both YIGSR and PHSRN allowed cell attachment, no cell spreading was observed on these surfaces. On RGD/YIGSR surfaces, the migration of EC was significantly higher than on RGD grafted PEG. The addition of PHSRN altered neither the adhesion nor the migration of EC.

In fibronectin molecules, both oligopeptides RGD and PHSRN are spaced by about 40 Å. The same distance was preserved in RGDG$_{13}$PHSRN that was grafted on PEG. RGDG$_{13}$PHSRN significantly increased the attachment, proliferation, metabolic activity and alkaline phosphatase production of rat calvarial osteoblasts compared to RGD or PEG or scrambled RDGG$_{13}$HPRNS; the cell spreading on both oligopeptides was improved compared to unmodified PEG (Benoit *et al*. 2005). The cells on both oligopeptides had a large number of focal adhesion plaques and a well-developed actin-cytoskeleton. Interestingly, cells growing on an unmodified PEG surface and scrambled RDGG$_{13}$HPRNS showed significantly higher synthesis of extracellular matrix, while in the cells on RGD, the synthesis was

moderate, and the lowest amount of ECM was found on $RGDG_{13}PHSRN$. A possible explanation for this is that the peptide-grafted PEG substrates acted as analogues of the natural ECM, and consequently the receptor-ligand interaction may lead to signal transduction that down-regulates ECM production. However, sufficient ECM production is necessary for bone regeneration, where especially biodegradable scaffolds must be replaced by ingrowth of the newly formed bone tissue (Benoit *et al*. 2005).

To increase the availability of grafted oligopeptides for cells, precise orientation, surface density and spacing of the ligands are essential for their effect (Aota *et al*. 1994). The minimum spacing of GRGDY was 440 nm for spreading and 140 nm for focal contact formation of human foreskin fibroblasts. Increasing the concentration of GRGDY from 10^{-3} fmol/cm^2 to 1 fmol/cm^2 significantly supported cell spreading (Massia and Hubbell 1991). A further increase in GRGDY concentration in the range of 10-100 fmol/cm^2 improved the formation of focal contacts. Another study with adsorbed fibronectin and fibronectin cell binding fragment obtained a spacing of 10 nm for maximum spreading on fibronectin, and a spacing of 12 nm for the cell binding fragment (Humphries *et al*. 1986; Massia and Hubbell 1991). The great difference between the concentrations in these studies may be due to a different protein orientation during its adsorption, making cell binding domains inaccessible for cells; alternatively, the altered three-dimensional conformation of the protein reduced its affinity for integrin receptors. Similarly, platelet activation on fibrinogen adsorbed on poly(alkyl metacrylate) correlated positively with the portion of fibrinogen recognizable by the antifibrinogen specific antibody but not by the total amount of bound fibrinogen. This resulted from a different fibrinogen conformation on various poly(alkyl metacrylate) surfaces (Lindon *et al*. 1986).

Another way of functionalizing synthetic materials is to immobilise cell growth factors and hormones, or their functional parts. For example, insulin alone, or together with RGDS, was grafted on the surface of a thermoresponsive polymer (Hatakeyma *et al.* 2006). The RGDS/insulin grafted surface enhanced EC growth significantly compared to RGDS or insulin alone. A lower amount of immobilised insulin compared to its soluble form was needed to achieve the same EC proliferation. Immobilised insulin molecules are known to stimulate their receptors and downstream transduction proteins, e.g. phosphatidylinositol-3-kinase, without internalization of ligand-receptor complexes (Ito *et al*. 1998).

An important feature of synthetic ECM-derived adhesion ligands, which can be advantageously utilized in tissue engineering, is that some of these ligands are recognized preferentially by specific integrin molecules, and thus by certain cell types. For example, the

integrin receptor $\alpha_4\beta_1$ is present in endothelial cells, but not in platelets. An adhesion ligand specific for this receptor, REDV, present in fibronectin, can be used for its selectivity to attract endothelial cells preferably to other cells types (Hubbel 1999). Another peptide sequence, KRSR, binds the transmembrane proteoglycans, e.g. heparin sulphate, of osteoblasts, and can thus be used for functionalizing the surface of bone prostheses (Balasundaram and Webster 2006).

1.7. Micropatterned surfaces

As mentioned above, in native blood vessels, smooth muscle cells have a quiescent contractile phenotype connected with their primary function: the contraction and dilation of the blood vessel wall to regulate blood pressure and flow. They have an elongated morphology, are aligned in a circumferential direction, and have increased expression of VSMC contractile proteins. When cultivated *in vitro*, VSMC quickly convert their phenotype to the synthetic and proliferative phenotype characterised by a polygonal shape without an organized structure (Thakar *et al*. 2003; Williams 1998). It is known that the cell function is regulated by cell-cell and cell-matrix interactions, humoral functions, local chemical conditions, and mechanical forces (Stegemann *et al*. 2005). The cell phenotype is influenced by the occupancy of a set of integrins that bind to ECM. The ECM components, such as type IV collagen, laminin and elastin are able to either inhibit VSMC dedifferentiation *in vitro* or even start their redifferentiation (Thyberg and Hultgardh-Nilsson 1994; Goessl *et al.* 2001). However, when cultured on collagen or fibronectin, smooth muscle cells undergo a phenotypic change within 3 days (Yamamoto *et al*. 1993).

Micropatterned surfaces are composed of both adhesive and antiadhesive microdomains and usually allow selective cell attachment to the adhesive microdomains. Both the shape and size of the discrete adhesive microdomains greatly influence the behaviour of the attached cells. This led to a hypothesis that by controlling the cell morphology in terms of the shape and size of the microdomains we would be able to regulate cell differentiation.

On strip-like domains of collagen from 20 to 30 μm in width, the VSMC had an elongated morphology and decreased proliferation compared to the control collagen surface. In addition, the VSMC had reduced both the number of α-actin filaments and α-actin expression in the strips, and were less stained for N-cadherin. VSMC cultivated in 30-μm-wide grooves of topographically micropatterned PLGA showed a similar morphology, proliferation and actin assembly as on collagen strips (Thakar *et al*. 2003). The minimum

adhesion area for the survival of individual VSMC was found to be 400 μm^2 (Goessl *et al*. 2001), and the area of well-spread VSMC on an unpatterned surface was about 2000 μm^2. The cells growing on 10 x 100 μm^2 areas spread in a spindle-like form. The actin filament bundles were found mainly aligned around the edges of the square-patterned areas or parallel to the long sides of oblong areas with strong polarization of the cytoskeleton. Some cells were able to span up an area of 10 x 100 μm and stretched to a length of 620 μm. Cell shape and size also influenced the shape and size of the cell nucleus, because the nuclei are mechanically connected to the cytoskeleton. A large number of VSMC on the microdomains grew in agglomerates partially overlapping each other; the cells did not maintain their individual territories, which is typical for a contact-inhibited confluent cell layer (Goessl *et al*. 2001). These results suggested that restricted spreading at least in one direction had a significant impact on cell proliferation and the assembly of actin cytoskeleton. In smooth muscle differentiation, small GTPase RhoA activation can be a convergent point for multiple factors, such as integrin-matrix interactions, mechanical stretch, endothelin-1, and angiotensin II, which are known to regulate the expression and differentiation of smooth muscle cell-specific genes (Mack *et al*. 2001).

Endothelial cells, cultured on spherical beads coated with fibronectin, spread and grew when the diameter of the beads was above 100 μm; whereas on 25 μm beads, the cells extended around the beads and less than 10 % of the cells underwent apoptosis. On beads with a diameter of 10 μm, the cell became rounded and the apoptotic index increased up to 60%, similar to that of nonadherent cells (Chen *et al*. 1997). On a square-shaped island coated with fibronectin, a progressive decline of apoptosis was observed if the island area increased from 75 to 3000 μm^2. This was accompanied by increased cell and nuclear spreading as well as DNA synthesis. In similar experiments with EC seeded on closely-spaced multiple adhesive islands 3-5 μm in diameter, it was confirmed that DNA synthesis scaled in direct proportion to the total projected area of the cells and not in proportion to the area of cell-ECM contacts (Chen *et al*. 1997).

Another possible explanation for the correlation between spreading and viability of cells is that differing cell morphology changes the tension distribution inside the cells (Huang and Ingber 1999; Goessl *et al.* 2001). The increased flexibility of the cytoskeleton observed in rounded cells may cause lethal intracellular structural arrangements including structural degeneration of the cell and nucleus during apoptosis (Chen *et al*. 1997). Cell survival is more tightly associated with cell shape in those cells that adhere to their substrate by integrins β_1 compared to integrins $\alpha_v\beta_3$; this finding is in agreement with the fact that β_1 integrins provide

a stronger anchor for resisting the cytoskeletal tension. From this perspective, micropatterned surfaces with an approppriate size of adhesive domains can prevent cell death and promote cell growth by resisting the contractile forces transmitted across integrins, thereby mechanically stabilizing the nucleocytoskeletal lattice (Wang *et al*. 1993; Chen *et al*. 1997).

In a three-dimensional collagen matrix, VSMC have been observed to have an elongated morphology, fewer actin fibres, lower α-actin expression, a lower proliferation rate, and enhanced ECM production (Stegemann and Nerem 2003; Thakar *et al.* 2003; Sarkar *et al*. 2005). VSMC cultured on three-dimensional micropatterned collagen scaffolds with 48 μm wide grooves were elongated and grew along the grooves regardless of the presence of pores (Sarkar *et al*. 2006). Since an elongated morphology was not sufficient to promote a contractile phenotype, other factors involving cyclic strain are necessary (Reusch *et al*. 1996). Cyclic strain increased synthesis of collagen, hyaluronate and chondroitine-6-sulfate, as well as expression of smooth muscle myosins in arterial VSMC. In addition, the strain also stimulated VSMC proliferation by increased production and autocrine action of PDGF (Reusch *et al*. 1996).

EC seeded on either pure or microtextured polyurethane with channels 95 μm in width and 32 μm in depth were exposed to shear stress from 10 to 60 dynes/cm^2 (Daxini *et al*. 2006). EC growing on microtextured material were significantly more resistant to detachment upon shear stress. The average shear stress in the channels was lower by 28% than on unmodified polyurethane. Microstructured surfaces prevented the denudation of large areas of the surface. Retention of the EC lining on a vascular prosthesis is essential for its antithrombotic functions. Interestingly, endothelial cells can favourably affect the function of a vascular graft, even if they are on the adventitial surface of the vessel and not in contact with the blood flow (Nugent and Edelman 2001). Gelfoam absorbable bovine collagen sponges seeded with EC and wrapped around the carotid arteries after balloon angioplasty showed significantly decreased restenosis and thrombosis compared to empty Gelfoam or arteries without perivascular wrapping. Implants containing xenogeneic cells reduced the restenosis by 31%, but those with allogeneic EC reduced the restenosis by 56% 12 weeks after injury, but both allogeneic and xenogeneic EC reduced occlusive thrombosis to a similar extent. At 90 days, the injured arteries in all groups were completely reendothelialized, so further intimal proliferation is not expected to occur. This can be explained by the finding that EC-derived compounds of low molecular weight can diffuse through the blood vessel and affect distant sites (Lovich and Edelman 1995).

1.8. Three-dimensional scaffolds

An advanced approach to tissue engineering is represented by the arrangement of artificial materials, especially resorbable materials, into three-dimensional porous scaffolds enabling the ingrowth and maturation of cells, and thus gradual replacement of the artificial cell carrier by the regenerated tissue. Prior to surgery, the scaffolds can be colonized by the patient's own cells, obtained bioptically and expanded in cell culture conditions. If cells of a specific tissue are lacking, the desired cell types can be obtained by seeding and differentiation of mesenchymal stem cells, obtained e.g. from the bone marrow, which are capable of differentiating into several cell types, including vascular endothelial cells, smooth muscle cells and osteoblasts (for a review, see Bacakova *et al*. 2004).

However, the growth of tissue inside the scaffolds is restricted by the diffusion of oxygen, which is limited to a depth of 100-200 μm in tissue (Hsiong and Mooney 2006). A sufficient vasculature is, therefore, essential to supply the newly formed tissue with oxygen, nutrients, osteogenic factors and stem cells, and also to eliminate waste products. New vessels arise either from vasculogenesis or angiogenesis. Vasculogenesis is referred as to *de novo* formation of blood vessels, in which EC and endothelial progenitor cells assemble to form vessels, while in angiogenesis existing vessels sprout to form new blood vessels (Davis *et al*. 2002, Hsiong and Mooney 2006; Price *et al*. 2004).

Cell growth as well as diffusion of nutrients and oxygen inside scaffolds is strongly influenced by scaffold porosity and permeability. Porosity, i.e. the volume percentage of void space in a material, usually ranges from 36% to more than 90%. The salt-leaching technique allows for the preparation of scaffolds with porosity of 70-90%, and allows regulation of scaffold porosity by the size of the porogen particles and the total volume fraction of the porogen (Chen *et al*. 2002; Zhao *et al*. 2002). The permeability of the scaffold, i.e. the cross sectional area of an effective channel for fluid to flow through the pore space, characterizes the pore interconnectivity of different scaffolds with similar porosities (Knackstedt *et al.* 2006; Sohier *et al*. 2003).

Both the size and the distribution of the pores inside the scaffolds influence the ingrowth of the various cell types. Porous ceramic pellets with pores between 44 and 100 μm in diameter were infiltrated *in vivo* preferably with connective tissue. In porous silicon nitride scaffolds, the endothelial cells attached preferentially to scaffolds with pores less than 80 μm, while the fibroblasts attached preferentially to scaffolds with pores larger than 90 μm. On collagen-glycosaminoglycan scaffolds, the attachment of MC3T3-E1 mouse osteogenic cells

increased proportionally to the specific surface area of the scaffold and inversely proportionally with the mean pore size when the pores were in the range of 96-150 μm (O'Brien *et al*. 2005). On the PLA scaffold, vascular smooth muscle cells grew preferably into pores ranging from 63 to 150 μm, fibroblasts preferred a pore size of 38-150 μm, and microvascular epithelial cells penetrated into pores larger than 38 μm (Zeltinger *et al*. 2001). The minimum pore size for osteoblast ingrowth is considered to be at least 100 μm (Simon *et al*. 2003). Substantial ingrowth of calcified bone tissue into the ceramic scaffold was visible between pore sizes of 100-150 μm (Hulbert *et al*. 1970). This corresponds to the approximate diameter of the normal Haversian system present in natural bone, which is between 100 and 200 μm (Karageorgiou and Kaplan 2005). In a wide range of pore diameters between 150 to 710 μm, the formation of mineralized bone tissue competed with the fast ingrowth of fibrovascular tissue into the available space that overtook osteoblasts in the PGLA foams within 7 days of cultivation (Ishaug-Riley *et al*. 1997).

An ideal highly-porous scaffold with interconnected pores should allow colonization throughout the scaffold, deposition and mineralization of the bone extracellular matrix and also graft vascularization. In practice, however, cells penetrate from the margins of the scaffold to a maximum depth of about 220-700 μm, due to the limited diffusion of nutrient and oxygen, and due to the accumulation of metabolic waste and material degradation products (Ishaug *et al*. 1997; Botchwey *et al*. 2003). Continuous cell growth from the margins of the defect with bridging in thin finger-like projections of bone is typical for the repair of empty defects. A wider distribution of pore sizes with large one-millimeter channels across the scaffold thickness allowed a discontinuous pattern of bone formation; cells were observed in multiple islands throughout the scaffold volume. The islands could later coalesce and fill the volume scaffold more quickly than in the continuous pattern (Simon *et al*. 2003).

Cell growth inside the scaffold can be improved under dynamic culture conditions in a bioreactor, such as a spinner flask, a rotating-wall system, and a perfusion cultivation system (Bilodeau and Mantovani 2006). Fluid perfusion secures efficient transport of gases, nutrients, catabolites and metabolites as well as stable pH of the medium. In addition, perfusion cultivation systems can mimic the mechanical stimuli to which EC are exposed in vessels, such as fluid shear stress, cyclic strain and hydrostatic pressure. EC respond to mechanical stimuli by reorganizing the actin containing stress fibres and other cytoskeletal components, by altering the metabolic and synthetic activities, and changing the cell cycle kinetics. Physiological fluid-mechanical stimuli could be important for EC differentiation and modulation of endothelial phenotype (Topper and Gimbrone Jr 1999).

1.9. Effects of shear stress and cyclic strain on cells *in vitro*

The endothelial cell lining in the blood vessels is often influenced by shear stress. The physiological response of EC to shear stress comprises a wide range of effects, such as regulation of endothelial release, activation of vasoconstrictors/vasodilators, adhesion molecules, paracrine growth factors, and mediators of inflammation and hemostasis (Resnick *et al*. 2003; McCue *et al*. 2003). To simulate shear stress, cells are usually cultivated in bioreactors with fluid flowing above the cells. Shear stress applied to EC cultured *in vitro* caused various effects, such as cell elongation, changes of cytoskeleton, and cell adhesion complexes. These physiological reactions of EC to shear stress reduce the impact of extremes of shear stress on EC (McCue *et al*. 2004). In subconfluent cultures *in vitro*, changes of cell morphology upon shear stress are driven by a cdc42-induced assembly of filopodia produced by the assembly of microfilament bundles, and by Rac-initiated lamellipodia produced by an assembly of dendritic arrays of F actin (Borisy and Svitkina 2000). Cdc42, Rac and Rho, all Ras-like molecules, belong to the GTPase superfamily of switch proteins that are involved in pathways activated by the binding of growth factors to receptor tyrosine kinases (Lodish *et al*. 2004). In confluent cell cultures, cell shape changes are driven by assembly and reorientation of basal stress fibres that insert into focal adhesion complexes and protrude plasma membrane at the upstream and downstream poles of the cells (Noria *et al*. 2004).

As for VSMC, these cells *in vivo* are embedded in the ECM of vessels, and are not exposed directly to the shear stress. In an *in vitro* experiment on a monolayer of VSMC, the shear stress either decreased or increased VSMC proliferation, induced FGF secretion, contraction, cytoplasmic calcium concentration, cell alignment, and apoptosis. Since VSMC proliferation in two-dimensional systems either decreased or increased upon cyclic tensile stress, it has been suggested that the proliferative response will depend on the initial phenotype of VSMC, which means that contractile cells proliferate whereas synthetic cells do not (Cappadona *et al*. 1999; for a review, see Stegemann *et al.* 2005).

In vessels, VSMC are exposed to cyclic tensile stress. Cyclic mechanical strain improved both the mechanical strength and the histological organization of VSMC-seeded collagen constructs, increased both collagen and elastin mRNA levels, and increased the expression of enzymes involved in tissue remodeling (Stegemann *et al.* 2005; Seliktar *et al*. 2000; Seliktar *et al*. 2003). It has been shown that mechanical stimulation is necessary to prevent transdifferentiation of smooth muscle cells toward the osteoblastic phenotype (Nikolovski *et al*. 2003; Stegemann *et al.* 2005). The extent of the applied mechanical strain also regulates

the responses of the VSMC. A zero or low strain, which is not physiological, causes a loss of the contractile phenotype; the optimal strain value supports a quiescent, contractile phenotype; and large strain results in hypertrophy, hyperplasia, increased matrix synthesis, hallmarks of the synthetic phenotype (Williams B 1998).

A shift of the VSMC phenotype can be a useful tool for producing a tissue engineered construct. VSMC of a synthetic phenotype can easily be expanded in cultures; they proliferate quickly after seeding, migrate and populate the polymeric scaffold, or they remodel protein matrices composed of fibrin or ECM proteins to produce a dense and mechanically robust tissue. In contrast, VSMC in an engineered graft should be in a contractile or quiescent state before implantation. Otherwise, synthetic VSMC can cause restenosis and failure of the graft (Williams 1998; Stegemann *et al.* 2005).

2. OBJECTIVES OF THE WORK

The goal of the work was to prepare and evaluate polymeric materials that could serve for inner modification of vascular prostheses or for creation of scaffolds for bone tissue regeneration. Thus, these polymers were expected to act as suitable matrices promoting the adhesion, proliferation and differentiation of vascular endothelial and smooth muscle cells or osteoblasts, which are prerequisites for the patency and proper functioning of vascular prostheses, as well as for the formation of functional bone tissue. Our hypothesis was that both natural (2.1. and 2.2.) and synthetic polymers (2.3. - 2.5.) are effective materials for these purposes.

2.1. The first aim of this study was to control the attachment, spreading growth and differentiation of vascular endothelial cells on synthetic and natural fibrin polymers. The reason was that these polymers could be used for modifying the inner surface of vascular prostheses in order to allow their endothelization and thus to improve their biocompatibility. In addition, thin fibrin assemblies can be used to modify the surface of the pore walls inside three-dimensional scaffolds without filling the entire volume of the pores with bulk fibrin gel. Therefore, we focused on the formulation of various fibrin assemblies of different thicknesses, and we assessed the impact of the thickness and structure of the gel on the adhesion, growth and differentiation of EC (Filová *et al*. 2008a; Brynda *et al*. 2008 [Czech patent application]).

2.2. Coating fibrin assemblies with ECM proteins can provide cells with further natural keys and mimic the natural cell environment. Therefore, the second aim of the study was to assess the influence of coating fibrin assemblies with type I collagen, fibronectin or laminin on adhesion, morphology, growth and differentiation of VSMC (Filová *et al*. 2005).

2.3. Artificial materials functionalized with short synthetic ligands for cell adhesion receptors have better defined and more controllable properties than natural polymers, including entire molecules of fibrin and ECM. Therefore, we studied VSMC colonization on synthetic analogues of ECM, which could be applied for reconstructing damaged vascular walls. In these analogues, a synthetic copolymer composed of PDLLA and PEO was chosen as an antiadhesive background to prevent uncontrollable cell attachment. The copolymer was grafted with a ligand for integrin receptors, i.e. oligopeptide GRGDS, and was tested in regard to its ability to support the adhesion, growth and differentiation of VSMC (Bačáková *et al*. 2007).

2.4. Three-dimensional PLGA scaffolds are considered as promising materials for the construction of advanced bioartificial bone prostheses. Therefore, we aimed to prepare synthetic biodegradable PLGA scaffolds with different pore sizes, and to evaluate the penetration, growth and differentiation of osteoblast-like cells inside the scaffold with regard to the method of scaffold preparation and its physical and chemical properties (Pamuła *et al.* 2008a, b).

2.5. Micropatterned surfaces are able to direct cell attachment and thus promote the regionally-selective cell colonization of the material on certain adhesive domains. The aim of the study was to prepare micropatterned surfaces containing microdomains made of acrylic acid or 1,7-octadiene and evaluate the attachment, adhesion, growth, and maturation of various cell types, including vascular smooth muscle cells, endothelial cells, mesenchymal stem cells and skeletal muscle cells on these surfaces (Filová *et al.* 2008b) .

3. MATERIALS AND METHODS

This dissertation is an interdisciplinary work that involves preparing material samples, testing their physicochemical properties and performing biological tests *in vitro*. This explains why the work has been done on the basis of cooperation among several of fields of natural sciences, such as chemistry, physics and biology. Since the thesis is based on five papers by the author, published or accepted in impacted international journals, six non-impacted papers, one invited review and one patent application, the materials and methods are decribed here only in an abbreviated form, and the details are provided in the attached author's publications.

3.1. Preparation and characterization of fibrin assemblies

Fibrin assemblies were prepared in cooperation with RNDr. Eduard Brynda, CSc. and his co-workers from the Institute of Macromolecular Chemistry, Acad. Sci. CR, Prague, and the Institute of Haematology and Blood Transfusion, Prague. Fibrin assemblies were prepared by successive incubation of the surface of non-plasma treated polystyrene or discoid PLLA fibrous blocks with fibrinogen and thrombin solutions. Two-dimensional assemblies were created using antithrombin-independent inhibitors of thrombin, i.e. D,L-prolyl-L-arginyl chloromethyl ketone and hirudin. Three-dimensional assemblies were prepared using inhibition with antithrombin III and heparin or without thrombin inhibitors (bulk Fb) (Filová *et al.* 2008a). Bulk fibrin assemblies were further coated with fibronectin, laminin or collagen I or mixed with collagen I. (Filová *et al*. 2005). PLLA blocks coated with fibrin were further stained with fluorescein isothiocyanate isomer I and observed under a Leica confocal microscope (TCS SP2, Germany). The structure and physical and chemical properties of fibrin assemblies were evaluated by surface plasmon resonance, transmission electron microscopy, scanning electron microscopy, and atomic force microscopy (Filová *et al.* 2008a).

3.2. Preparation of PDLLA-PEO copolymers

Poly(lactic acid), PDLLA-PEO, and GRGDSG-PEO-b-PDLLA copolymers were synthesized by a ring-opening polymerisation of L- or D,L-lactide, respectively (Bacakova *et al.* 2007, Kubies *et al.* 2000, Rypáček *et al.* 2001). Thin films of these compounds were cast on glass coverslips, and their physical and chemical properties, such as wettability and molecular weight, were characterized in cooperation with RNDr. František Rypáček, CSc, and

his co-workers from the Institute of Macromolecular Chemistry, Acad. Sci. CR, Prague (Bacakova *et al.* 2007).

3.3. Preparation of PGLA scaffolds

The PGLA scaffolds were produced by a solvent casting/particulate leaching technique and their physical and chemical characteristics, such as measurement of the scaffold microstructure using a scanning electron microscope, evaluation of the compressive strength and compressive modulus of the scaffolds, scaffold porosity and permeability were measured (in detail described in Pamuła *et al.* 2008a,b) in cooperation with Dr. Elżbieta Pamuła, University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Krakow, Poland.

3.4. Preparation of micropatterned surfaces

Micropatterned surfaces were prepared by polymerisation of acrylic acid and 1,7-octadiene in a plasma reactor, and physicochemical properties of the surfaces were measured by X-ray photoelectron spectroscopy and wettability testing in cooperation with Dr. Alex Shard and his co-workers from the Department of Engineering Materials, University of Sheffield, Sheffield, UK (in detail described in Filová *et al.* 2008b).

3.5. Cells and culture conditions

The fibrin structures were seeded with endothelial cells (EC) (line CPAE ATCC CCL-209, from bovine pulmonary artery, Rockville, MA, U.S.A) at the density of 16000 cells/cm^2 and 1.5 ml of Minimum Essential Eagle Medium supplemented with 2mM L-glutamin, Earle's BSS with 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, 20% of fetal bovine serum (FBS) and 200 U/ml aprotinin (in detail described in Filová *et al.* 2008a).

Fibrin assemblies coated with ECM proteins and also the PDLLA-PEO, and GRGDSG-PEO-b-PDLLA copolymers were seeded with vascular smooth muscle cells (VSMC) at a density of 18000 cells/cm^2 in 1.5 ml of Dulbecco-modified Eagle medium supplemented with 10% of FBS and 40 μg/ml of gentamicin or serum-free DMEM with ITS liquid media supplement containing insulin, transferrin and selenium (Sigma, St. Louis, MO, U.S.A., Cat. No. I-3146). VSMC were derived from the thoracic aorta of 8-week-old male Wistar rats by explanation method (Filová *et al*. 2005; Bacakova *et al.* 2007).

The PLGA scaffolds were seeded with human osteoblast-like cells of the line MG 63 (European Collection of Cell Cultures, Salisbury, UK). The cells were suspended to a concentration of 45,000 and 100,000 cells/mL in the Dulbecco-modified Eagle Minimum Essential Medium (DMEM; Sigma, U.S.A., Cat. N^{o} D5648) supplemented with 10% fetal bovine serum (FBS; Sebak GmbH, Aidenbach, Germany) and gentamicin (40 μg/ml, LEK, Slovenia). The cells were cultured for 15 days at 37^{o}C in a humidified air atmosphere containing 5% of CO_2 (Pamuła *et al.* 2008a,b).

The isolation and characterisation of mesenchymal stem cells was performed by Mgr. Jana Hlučilová and Mgr. Jiří Klíma, PhD at the Institute of Animal Physiology and Genetics, v.v.i., Acad. Sci. CR, in Liběchov, Czech Republic (in detail described in Filova *et al*. 2008b).

All other biological experiments involved in this dissertation, such as cell cultivation, measurement of cell growth and viability, evaluation of molecular markers of cell adhesion and differentiation by immunofluorescence staining and enzyme-linked immunosorbent assay, and other biochemical and immunocytochemical methods were performed at the Institute of Physiology, Acad. Sci. CR, and are described in detail in the author's publications.

3.6. Cell morphology, number, growth curves and doubling time

The shape of endothelial and smooth muscle cells, their number (day 1 and 3 after seeding), and the size of the cell spreading area (day 1 after seeding) were evaluated in hematoxylin and eosin stained cells on microphotographs taken under a phase-contrast microscope (Opton Axioplan, Germany), equipped with DSC-F707 digital camera (Sony Corp., Japan), and using the image processing software Atlas (Tescan s.r.o., Czech Republic) (in detail described in Bačáková *et al*. 2007). On day 7 after seeding, and in experiments with MG 63 cells in PGLA scaffolds, the cells were harvested with a trypsin-EDTA solution in PBS, and their numbers were measured using a Burker haemocytometer (Pamuła *et al.* 2008a,b). Cell densities per cm^2 were used for constructing the growth curves and for calculating the cell population doubling time on the tested surfaces. The doubling time (DT) was calculated using the following equation: $DT = \log 2\ (t\text{-}t_o)/(\log N_t\text{-}\log N_{to})$, where t_o and t represent earlier and later time intervals after seeding, respectively, and N_{to} and N_t the number of cells at these intervals (Pamuła *et al.* 2008a,b).

3.7. Laser confocal microscopy

The penetration of cells into the pores of PGLA scaffolds was evaluated in a laser confocal microscope (Leica TCS SP2, Germany), using horizontal optical sections through

the scaffolds every 20 μm up to a maximum depth of 1000 μm. For each type of scaffold, two samples were scanned using 1-2 scan series per sample (objective 10x). For confocal microscopy, the cells were visualized by staining with propidium iodide or by immunofluorescence staining against beta- actin (Pamuła *et al.* 2008a,b).

3.8. Immunoflurescence staining of alpha$_v$-integrins, CD 44, talin, vinculin, von Willebrand factor, alpha-actin and beta-actin

The cells were fixed in 2% paraformaldehyde in PBS for 5 min (pH 7.4), washed three times in PBS (5 min), pre-treated with 1% bovine serum albumin (BSA) in PBS containing 0.1% Triton X-100 (1 hour), then incubated in 1% Tween 20 for 20 min. After 5 min washing in PBS, the primary antibodies, i.e. rabbit anti-integrin α_v subunit polyclonal antibody, purified rat anti-pig CD44H, mouse monoclonal anti-talin or anti-vinculin antibodies, rabbit anti-human von Willebrand factor, monoclonal anti-alpha smooth muscle actin (mouse IgG2a isotype) or monoclonal anti-beta-actin, mouse ascites fluid, were applied overnight at 4ºC. As secondary antibodies, a goat anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugate, goat anti-mouse IgG (Fab specific) FITC conjugate, Alexa Fluor®488-conjugated (F(ab`)2 fragment of goat anti-mouse IgG (H+L), or Alexa Fluor®488-conjugated goat anti-rat IgG (H+L) (Molecular Probes), were used for 80 min at room temperature. The cells were evaluated under an epifluorescence microscope (Olympus IX51, digital camera DP70, Japan) or a Leica confocal microscope (TCS SP2, Germany) (Bačáková *et al.* 2007; Filova *et al.* 2008a,b; Pamula 2008a,b).

3.9. Enzyme-linked immunosorbent assay (ELISA)

The concentrations of alpha$_v$-integrins, CD 44, focal adhesion proteins talin and vinculin, von Willebrand factor, alpha-actin, beta-actin, osteopontin and osteocalcin were measured per mg of protein in the cells after three-day or seven-day cultivation (Filová *et al.* 2008a; Pamuła *et al.* 2008b).

. The cells were detached by trypsin-EDTA. For more effective digestion of the fibrin structures and cell detachment, the trypsin-EDTA solution was used at a concentration 2.5 times higher than that recommended by the manufacturer. The cells were then resuspended in PBS, centrifuged, again resuspended in PBS (10^6 cells/ml), and kept in a freezer at -70°C overnight. The cell homogenates were then prepared by ultrasonication for 10 seconds using a sonicator and the total protein content was measured using a modified method by Lowry. Aliquots of the cell homogenates corresponding to 1–50 μg of protein in 50 μl of water were

adsorbed on 96-well microtiter plates at 4°C overnight. After washing twice with PBS, the non-specific binding sites were blocked by 0.02% gelatine in PBS (60 min). The primary antibodies, such as polyclonal rabbit anti-human integrin alpha$_{v,}$ purified rat anti-pig CD44H, mouse monoclonal anti-talin or anti-vinculin antibodies, rabbit anti-human von Willebrand factor, monoclonal anti-alpha smooth muscle actin (mouse IgG2a isotype), monoclonal anti-beta-actin, mouse ascites fluid, clone AC-15, rabbit anti-osteocalcin polyclonal antibody, and rabbit polyclonal antibody to osteopontin, were diluted in PBS and applied for 60 min at room temperature. As secondary antibodies, we used goat anti-rabbit IgG, goat anti-rat IgG polyclonal antibody, or anti-mouse IgG (Fab specific) antibody. All secondary antibodies were conjugated with peroxidase and applied for 45 min at room temperature. This step was followed by double washing in PBS with Triton X-100 (0.1%) and orthophenylendiamine reaction (Sigma, 2.76 mM) using 0.05% H_2O_2 in 0.1 M phosphate buffer (pH 6.0, dark place). The reaction was stopped after 10–30 min by 2M H_2SO_4 (50 µl/well) and the absorbance was measured at 492 nm by Wallac VICTOR^2, 1420 Multilabel Counter.

3.10. Evaluation of DNA synthesis by a colorimetric method

The studied materials were seeded with MG 63 cells in the density of 20 000 cells/well at the density of 20 000 cells/well. On day 5 after seeding, DNA synthesis in cells was evaluated by a commercially available Cell Proliferation ELISA BrdU (Colorimetric) kit (Roche s.r.o., Diagnostic Division) according the manufacturer's protocol. Briefly, the cells were incubated with 5-bromo-2-deoxy-uridine (BrdU) labeling reagent (4 h), FixDenat solution (30 min), Anti-BrdU POD solution (90 min), washing solution (3 x 2 min), substrate solution (10 min, dark place), and finally STOP solution (1M H_2SO_4) was added. The resulting solution was then moved into fresh plates and the absorbance was measured using Wallac Victor 1420 Multilabel Counter at the wavelength of 450 nm (the reference value was 690 nm) (Pamuła *et al.* 2008b).

3.11. Evaluation of DNA synthesis by immunocytochemical staining

VSMC were incubated with 40 µM BrdU (30 min), washed with PBS, fixed with a 70% ethanol (-20°C) for 15 min, and their DNA was partially denatured by treatment with 3N HCl (20 min). The samples were washed in 0.1 M sodium tetraborate (pH 8.5, 10 min) and non-specific binding sites were blocked by 3% bovine foetal serum in PBS with 0.3% Triton X-100 (Sigma, St. Louis, MO, U.S.A.) for 30 min. The newly synthesised DNA was

immunolabelled with an anti-BrdU mouse monoclonal antibody (Exbio, CR). Biotin-conjugated goat anti-mouse IgG (Fab specific, Sigma) and ExtrAvidin-Peroxidase (Sigma) were used in consequent steps. All three chemicals were diluted in PBS with 3% bovine foetal serum and 0.3% Triton X-100 (60 min, 20ºC). As chromogen, 3, 3'- diaminobenzidine was used, the colour was intensified by $NiCl_2$ (5 min). The cells were counterstained with light green and the percentage of BrdU-labelled nuclei was assessed in phase contrast microscope. For each experimental group, 10 randomly chosen microscopic fields were evaluated (Filová *et al*. 2008b).

3.12. MTT assay

The growth and viability of cells colonizing the PLGA scaffolds were also evaluated by measuring mitochondrial dehydrogenase activity using a modified MTT(3-(4,5-dimetyl-2-tiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay. Briefly, the cells on day 7 after seeding on the scaffolds were incubated with 150 µl DMEM and 50 µl of 0,2 % MTT (Sigma, Cat. No. M5655) in PBS for 4 h. Then, 90 µl of 0.69 M sodium dodecyl sulphate in 50% *N,N*-dimethylformamide (pH 4.7) was added and incubated for 3 hours. Then, after stirring 8 times, the resulting solution was moved into fresh plates and the absorbance was measured using ELISA reader TECAN Spectra A5082 (TECAN, Austria) at the wavelength of 570 nm (the reference value was 690 nm). Two types of control samples were used: (1) scaffolds with cells in medium without MTT (background) and (2) scaffolds without cells in medium with MTT (blank) (Pamuła *et al*. 2008a).

3.13. Statistical analysis

The quantitative data was presented as mean ± SEM. 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 $p \leq 0.05$ was considered significant.

4. RESULTS

4.1. Endothelial cells on fibrin assemblies

4.1.1. FILOVÁ E., BRYNDA E., RIEDEL T., BAČÁKOVÁ L., CHLUPÁČ J., LISÁ V., HOUSKA M., DYR J.E.: Vascular Endothelial Cells on Two- and Three-Dimensional Fibrin Assemblies for Biomaterial Coatings. *Journal of Biomedical Materials Research. Part A*. J Biomed Mater Res A. 2008a May 15 [Epub ahead of print], impact factor 2.612

4.1.2. BRYNDA E., RIEDEL T., DYR J., HOUSKA M., BAČÁKOVÁ L., FILOVÁ E., CHLUPÁČ J., LESNÝ P., JENDELOVÁ P,, SYKOVÁ E. Způsob přípravy regulovaných vrstev fibrinu na pevných površích*, Czech Patent No 299687, Application No. 2006-821, registered in the UPV Office on Dec 21, 2006, accepted on September12 2008.*

Both publication and patent deal with adhesion, growth and differentation of endothelial cells on fibrin assemblies of different thicknesses from ultrathin two-dimensional to thick bulk fibrin gel. Two-dimensional fibrin structures were obtained by repeated, successive adsorption of fibrinogen, incubation of surface with thrombin solution, and inhibition of surface attached thrombin by D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone and hirudin. Three-dimensional fibrin networks immobilized at the surface were formed by catalytic action of surface-attached thrombin on the ambient fibrinogen solution. Thin three-dimensional fibrin networks were obtained if thrombin inhibitors antithrombin III and heparin were added into a fibrinogen solution. These techniques enable the inner surface of synthetic scaffolds to be coated with fibrin films without filling their pores with bulk fibrin gel (Fig. 1). The growth of EC seeded on a polystyrene surface coated with Fb films was evaluated by the number and morphology of the adhering EC and concentration of beta-actin, vinculin and von Willebrand factor. The best initial cell spreading after 1 day was observed on two-dimensional fibrin and thin three-dimensional fibrin. The highest concentration of von Willebrand factor, a marker of EC differentiation, and $alpha_v$-intergrins were observed after 3 days on thick three-dimensional fibrin (Fig. 2). The highest EC population densities after 7 days were observed on two-dimensional and thick three-dimensional fibrin layers. Fibrin layer on the surface of vascular prostheses can be prepared as autologous material from patient's blood and immune reaction can be avoided (see the objective 2.1.).

Fig. 1.

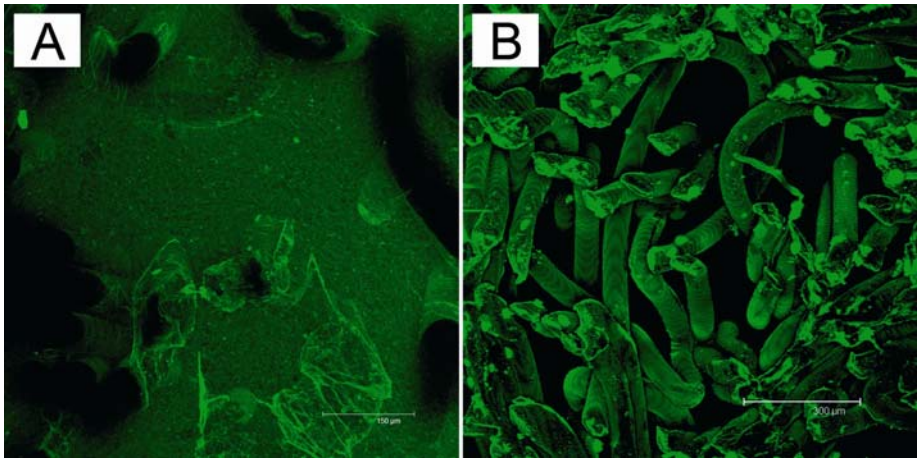


Fig. 1. Fluorescence staining of fibrin with FITC in a polylactide scaffold filled with (A) bulk Fb gel and (B) modified with thin three-dimensional Fb gel. Confocal microscope (Leica TCS SP2, Germany), obj.×20 (A) and ×10 (B), scale bar 150 μm (A) and 300 μm (B).

Fig. 2.

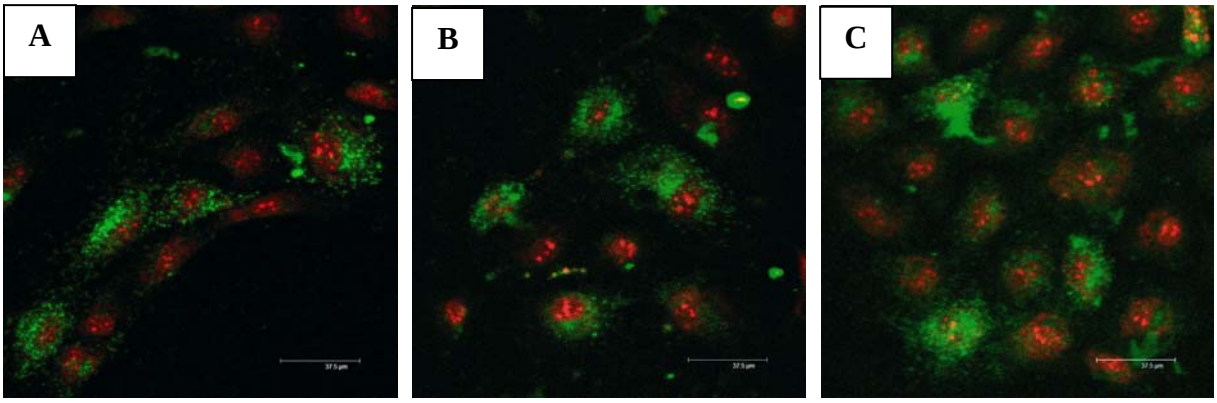


Fig. 2. Immunofluorescence staining of von Willebrand factor in endothelial cells cultured on two-dimensional (A), thin three-dimensional (B) and thick three- dimensional fibrin assemblies. Confocal microscope Leica, obj. ×20, zoom ×4, scale bar 37.5 μm.

4.2. Vascular smooth muscle cells on fibrin assemblies modified with ECM proteins

4.2.1. FILOVÁ E., BRYNDA E., HOUSKA M., RIEDEL T., BAČÁKOVÁ L.: Adhesion and growth of vascular smooth muscle cells on protein assemblies for biomaterial coating. *Eng Biomater (Inzynieria Biomaterialów)* 8:(47-53): 9-12, 2005; a newly established journal not yet with an impact factor.

In this study, we investigated the growth of vascular smooth muscle cells (VSMC) on fibrin assemblies coated with ultrathin layer of collagen I, fibronectin or laminin. We observed improved initial attachment and spreading of VSMC compared to polystyren. The adhesion area of VSMC increased on fibrin coated with collagen I and fibronectin, while on laminin it remained similar as that on the pure fibrin. Decreased DNA synthesis of VSMC was found on fibrin coated with laminin on day 3 after seeding. VSMC proliferation increased on pure fibrin, and on fibrin coated with either fibronectin or type I collagen after 7-day cultivation. Thin three-dimensional fibrin net markedly changed the morphology of VSMC from polygonal to star-like shape with protrusions. Cells all tested samples formed vinculin-containing focal adhesion plaques and well-developed α-actin cytoskeleton, an important marker of VSMC differentiation (Fig. 1). The protein assemblies can be used for improvement of cell adhesion and growth on various artificial materials used in tissue engineering, e.g. for construction of bioartificial vascular prostheses (see the objective 2.2.).

Fig. 1.

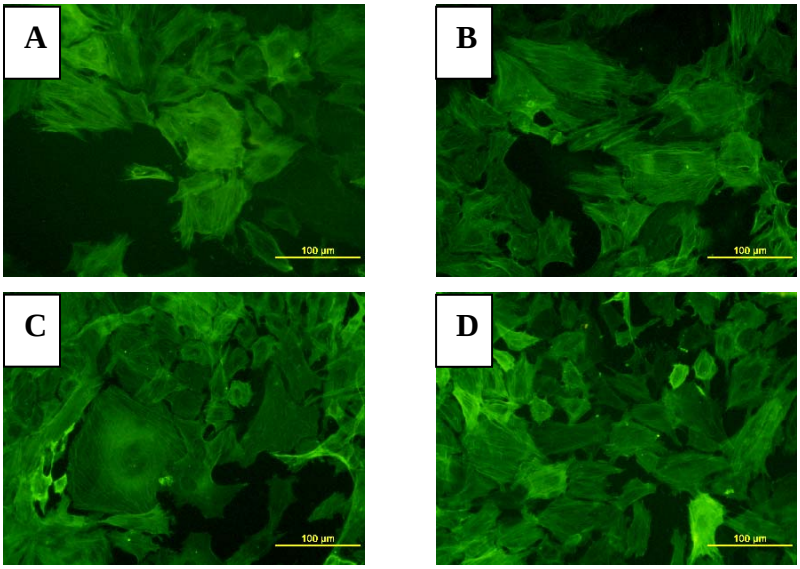


Fig. 1. Immunofluorescence staining of alpha-actin in VSMC cultured on fibrin (A), fibrin coated with laminin (B), collagen I (C) and fibronectin (D). Olympus IX51, camera DP70, obj. ×20.

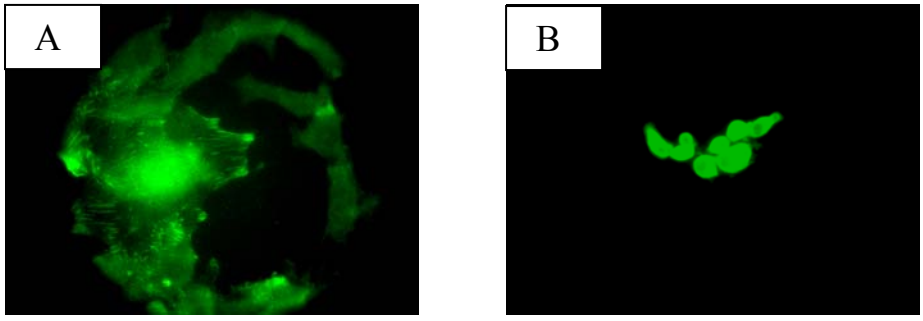
4.3. Vascular smooth muscle cells on polylactides grafted with adhesion oligopeptides GRGDSG

4.3. BACAKOVA L., FILOVA E., KUBIES D., MACHOVA L., PROKS V., MALINOVA V., LISA V., RYPACEK F.: Adhesion and Growth of Vascular Smooth Muscle Cells in Cultures on Bioactive RGD Peptide-Carrying Polylactides. *Journal of Materials Science: Materials in Medicine* 18(7):1317-23, 2007, impact factor 1.248.

In this study, we evaluated the adhesion and growth of vascular smooth muscle cells (VSMC) on foils prepared of poly(DL-lactide) (PDLLA), a block copolymer of PDLLA and polyethylene oxide (PDLLA-*b*-PEO) and PDLLA-*b*-PEO, in which 5% or 20% of the PEO chains were grafted with Gly-Arg-Gly-Asp-Ser-Gly (GRGDSG) oligopeptide.

In serum-supplemented media the number of initially attached to PDLLA (6 and 24 hours after seeding), their spreading and formation of vinculin- and talin- containing focal adhesion sites were similar as on standard cell culture polystyrene However, the copolymer of PDLLA and PEO was almost completely antiadhesive for cells, providing a good inert background for controlled attachment of ligands for cell adhesion receptors. Grafting of GRGDSG peptide to the copolymer restored the cell adhesion almost to the values found on PDLLA. Surprisingly, the concentration of 5% GRGDSG was usually more effective in promoting cell adhesion than the higher concentration of 20%. On the other hand, the higher GRGDSG concentration was slightly more active in stimulating DNA synthesis in VSMC. Similar results were obtained in serum-free media, although the receptor-mediated adhesion and growth of cells were generally lower than in serum-supplemented media. Nevertheless, these results indicate that an effective control of the adhesion and proliferation of VSMC cells on synthetic polymers could be established, and the artificial extracellular matrix analog could be used for vascular tissue repair and regeneration (see the objective 2.3.).

Fig. 1.



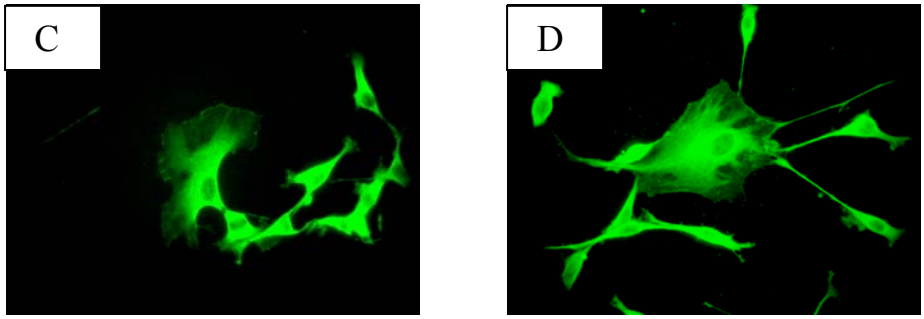


Fig. 1. Immunofluorescence staining of vinculin in VSMC cultured on PDLLA (A), PDLLA-b-PEO (B), PDLLA-b-PEO-5%GRGDSG (C) and PDLLA-b-PEO-20%GRGDSG (D). (Opton, Axioplan, Germany), oil immersion, obj. ×100.

4.4. Human osteoblast-like MG 63 cells on polymeric porous 3D scaffolds or bone tissue engineering

4.4.1. PAMUŁA E., BACAKOVA L., FILOVA E., BUCZYŃSKA J., DOBRZYNSKI P., NOSKOVA L, GRAUSOVA L..: The influence of pore size on colonization of poly(L-lactide-glycolide) scaffolds with human osteoblast-like MG 63 cells *in vitro. Journal of Materials Science: Materials in Medicine,* 2008a*,* 19(1): 425-435, impact factor 1.248.

4.4.2. PAMUŁA E., FILOVÁ E., BAČÁKOVÁ L., LISÁ V., ADAMCZYK D.: Resorbable polymeric scaffolds for bone tissue engineering: The influence of their microstructure on the growth of human osteoblast-like MG 63 cells. *Journal of Biomedical Materials Research. Part A,* 2008b Apr 22 [Epub ahead of print], impact factor 2.612.

In these studies, three-dimensional PLGA scaffolds with different pore sizes were prepared by solvent casting/particulate leaching technique. We investigated the adhesion, penetration, proliferation and differentiation of human osteoblast-like MG 63 cells cultured in scaffolds under static conditions. We found that increased pore size, rather than the technique used for preparing the scaffold, improved the osteogenic behaviour of MG-63 cells in the scaffolds (see the objective 2.4.). Three types of PLGA scaffolds were prepared, having the same high porosity (87 %) but increasing diameter of the pores (40, 200 μm, 400 - 600 μm) and increasing pore interconnectivity. As revealed by conventional fluorescence and confocal microscopy on days 5 and 7 after seeding, the cells on the scaffolds of large or medium pore size infiltrated the inside part of the material, whereas on the scaffolds of small pore size, the cells were retained on the material surface. On day 7 after seeding, the highest number of cells was found on the scaffolds of the largest pore, whereas on the scaffolds with medium and smallest pore diameter, the number of cells was almost three times lower and similar for both pore sizes. These results corresponded well with incorporation of bromodeoxyuridine into newly synthesized DNA, which was significantly higher in cells on the scaffolds of the largest pore size than on the material with medium and smallest pore diameter. As indicated by MTT test, the mitochondrial activity in cells on the scaffolds with medium pore size was similar to that on the material with the highest pore size and significantly higher than on the scaffolds of the smallest pore diameter. These results suggest that the PLGA scaffolds with the largest pore diameter (600 μm) and better pore interconnectivity are the most suitable for colonization with osteogenic cells (see the objective 2.4.).

Fig. 1

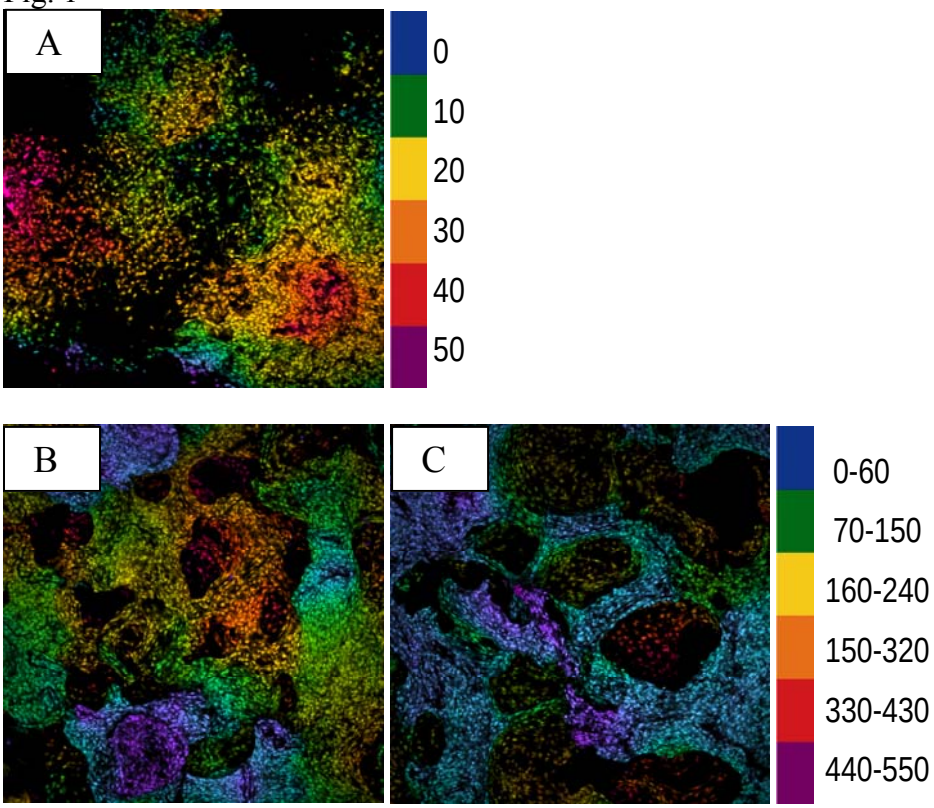


Fig.1. Osteoblast-like MG 63 cells growing in PGLA scaffolds with pore size of 40 µm (A), 200 µm (B) and 600 µm (C) on day 14 after seeding; cells penetrated into the depth of 40 µm (A), 580 µm (B) and 640 µm (C). Confocal miscroscope Leica SP2, obj. ×10.

4.5. Regionally-selective cell colonization of micropatterned surfaces

4.5.1. FILOVÁ E., BULLETT N.A., BAČÁKOVÁ L., GRAUSOVÁ Ľ., HAYCOCK J.W., HLUČILOVÁ J., KLÍMA J., SHARD A.: Regionally-selective cell colonization of micropatterned surfaces prepared by plasma polymerisation of acrylic acid and 1,7- octadiene. *Physiological Research*, accepted, impact factor 1.505.

Micropatterned surfaces have been used as a tool for controlling the extent and strength of cell adhesion, the direction of cell growth and the spatial distribution of cells. In this study, chemically micropatterned surfaces were prepared by successive plasma polymerisation of acrylic acid (AA) and 1,7-octadiene (OD) through a mask. Vascular smooth muscle cells (VSMC), endothelial cells (EC), or mesenchymal stem cells (MSC) were seeded on these surfaces in densities from 9,320 cells/cm^2 to 31,060 cells/cm^2. All cell types adhered and grew preferentially on the strip-like AA domains with different selectivity. Between day 1 and 7 after seeding, the percentage of cells on AA domains ranged from 84.5 to 63.3% for VSMC, 85.3 to 73.5% for EC, and 98.0 to 90.0% for MSC. The enzyme-linked immunosorbent assay (ELISA) revealed that the concentration of alpha-actin per mg of protein was significantly higher in VSMC on AA. Similarly, immunofluorescence staining of von Willebrand factor showed more apparent Weibel-Palade bodies in EC on AA domains. MSC growing on AA had better developed beta-actin cytoskeleton, although they were less stained for hyaluronan receptor (CD44). In accordance with this, MSC on AA contained a higher concentration of beta-actin, although the concentration of CD44 was lower. These results based on four cell types suggest that plasma polymerisation is a suitable method for producing spatially defined patterned surfaces for controlled cell adhesion, proliferation and maturation (see the objective 2.5.).

Fig. 1.

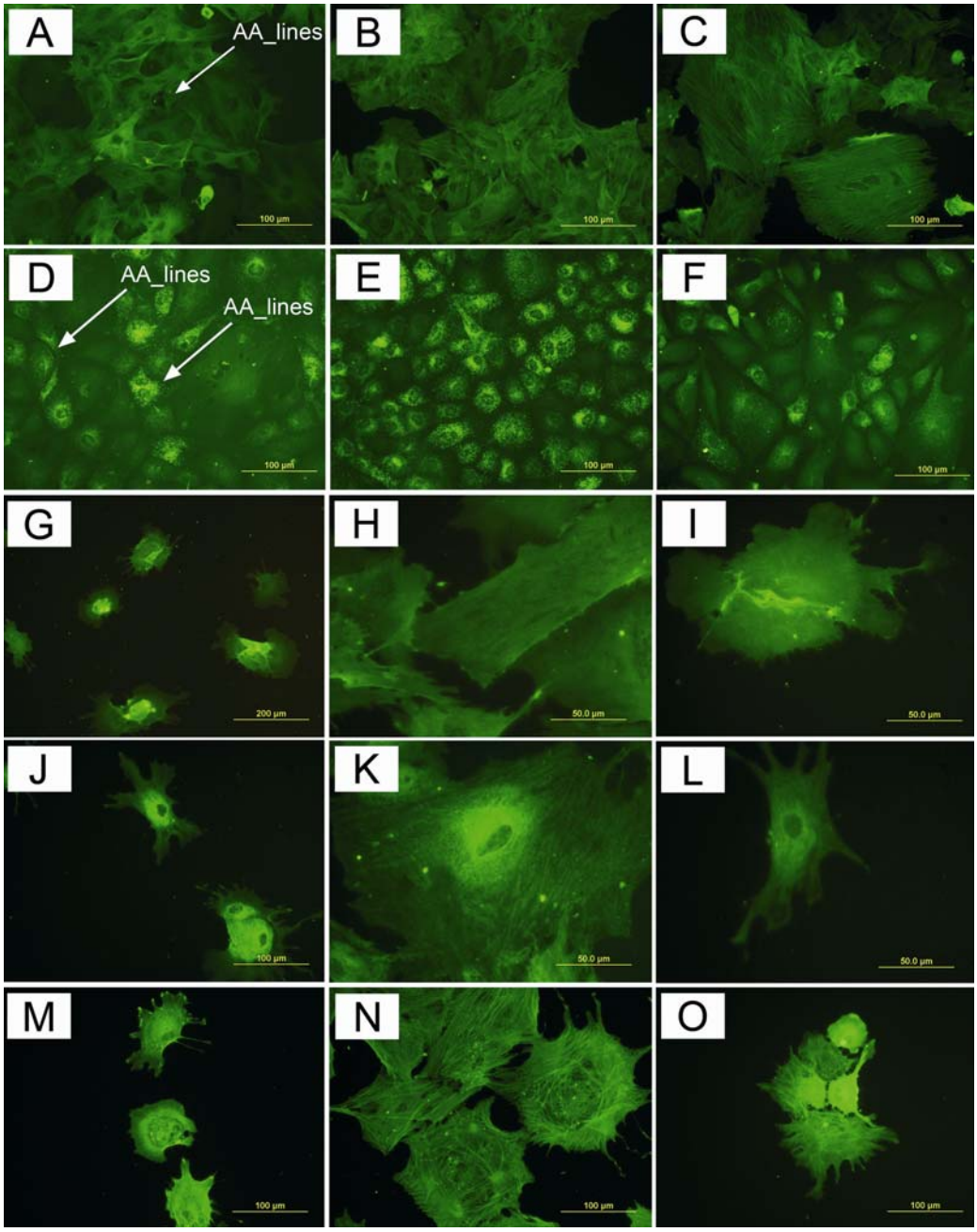


Fig. 1. Immunofluorescence staining of alpha-actin in vascular smooth muscle cells (A-C); von Willebrand factor in endothelial cells (D-F), CD44 (G-I), talin (J-L) and beta-actin (M-O) in mesenchymal stem cells; growing on domains composed of acrylic acid (A, D, G, J, M), pure acrylic acid (B, E, H, K, N), and pure 1,7-octadiene (C, F, I, L, O). Olympus IX51, digital camera DP70, obj.×20, bar = 100 μm (A-F, J, M-O); obj.×10 (G), bar = 200 μm; and obj.×40, bar = 50 μm (H, I, K, L).

5. DISCUSSION

In the study on fibrin assemblies, we have demonstrated that a variety of fibrin assemblies from ultrathin two-dimensional to thick three-dimensional bulk gels support adhesion, growth and differentiation of EC (Filová *et al*. 2008a; Brynda *et al*. 2008 [Czech patent application]). The first sealing method for knitted or woven vascular prostheses involved pre-clotting with the patient's blood before heparin injection (Yates *et al*. 1978). Later, various resorbable sealing matrices were used, such as albumin, gelatine and collagen, cross-linked with chemical reagents. However, these matrices did not to support healing. Sealing the prostheses with autologous proteins promotes healing by adding cellular and extracellular components to the matrix. In addition, autologous fibrin matrices can be used as carriers of growth factors or endothelial cells, and of bone marrow cells to enhance endothelialisation and reduce intimal hyperplasia (Cardon *et al*. 2000).

Two-dimensional fibrin structures were prepared using inhibitors of fibrin-bound thrombin, PPACK, and hirudin (Filová *et al*. 2008a; Brynda *et al*. 2008 [Czech patent application]). Surface fibrin gels were prepared using antithrombin III and heparin, which inhibited only free thrombin in solution. Bulk fibrin gels were prepared either from citrated human blood plasma supplemented with $CaCl_2$ or from pure fibrinogen and thrombin without any inhibitors. In our experiments, the thickness of the fibrin fibres increased proportionally to the thickness of the final fibrin assemblies. In agreement with Carr (Carr 1988), a bulk fibrin gel prepared from blood contained thicker fibres, and the network was less porous compared to fibrin prepared from fibrinogen and thrombin. The thickness of the fibres of fibrinogen prepared from fibrinogen and thrombin did not increase with increased fibrinogen concentration, but correlated inversely with thrombin concentration. This is in agreement with the observations of Blombäck and co-workers, who showed that fibrin networks formed at a low concentration of thrombin were more branched and tighter, i.e. less porous than networks formed at high levels of thrombin (Blombäck *et al*. 1994). This type of tight network contained a higher degree of equilateral fibril branching and was more elastic (Mosesson 2005). Rigid clots had long and thick fibrin fibre strands that constituted a stable and porous polymer, whereas malleable fibrin clots were less porous and had shorter and thinner fibrin strands. The structure and properties of a fibrin clot are strongly influenced by pH, ionic strength, and other biophysical parameters of the milieu in which they polymerized (Nehls and Herrmann 1996). A high salt concentration used during fibrin gel preparation resulted in a finer clot structure formation. On the gel with thinner fibres and smaller pores among them,

poor attachment and migration of fibroblasts were observed (Redl and Schlag 1985; Amrani *et al*., 2001). However, we observed similar numbers of initially adhered EC on day 1 after seeding on all studied fibrin assemblies. Nevertheless, the highest initial spreading of EC was found on two-dimensional and thin surface fibrin gel of low density. These cells had the largest cell-material contact area and well developed focal adhesion plaques containing integrin adhesion molecules with an $alpha_v$ chain. On the other hand, three-dimensional thin surface fibrin gel of high fibrinogen concentration did not allow good cell spreading until day three after seeding. EC growing on bulk fibrin gels were less spread, elongated with protrusions. EC rounding, which appeared to be necessary for capillary morphogenesis, is promoted by a decrease in integrin-matrix contacts, and also by a reduction in the tensile strength and the rigidity of the extracellular matrix (Nehls and Herrmann 1996). On the inner surface of artificial vascular prostheses, EC should be morphologically mature, i.e. well spread and flattened (Fields and Cassano 2002). If EC do not adhere tightly to a biomaterial surface, they are washed away when exposed to fluid shear stress. In particular, hydrophobic, non-sticky surfaces like ePTFE are prone to delamination of the EC lining upon implantation due to surgical, i.e. mechanical, manipulation, and subsequent exposure to blood shear stress (Walpoth and Bowlin 2005).

After 7 days of culture, the highest population density of EC was found on two-dimensional fibrin assemblies and on bulk gel prepared from fibrinogen and thrombin. The lowest cell densities were on thin surface fibrin gels. We suppose that fibrin degradation was not entirely stopped by the aprotinin used during cell cultivation, and gradual fibrin degradation could impair EC proliferation mainly on rather thin surface attached fibrin assemblies. On the other hand, firmly attached two-dimensional fibrin layers, crosslinked fibrin as well thick bulk gels seemed to be less susceptible to fibrinolysis. Surprisingly, the density of endothelial cells on bulk fibrin prepared from fibrinogen and thrombin was higher than that on fibrin from blood plasma after 7-day cultivation, although the concentrations of fibrinogen and thrombin were the same in both fibrin samples. We suppose that the fibrin gel produced from human blood plasma contained other compounds present in blood that interacted with cell growth. Thrombus that occurs after injury by activation of a coagulation cascade contains both platelets and fibrin, released growth factors, e.g. PDGF, or chemokines, proteolytic enzymes produced by neutrophils and monocytes. All these compounds are quickly incorporated into the clot. They affect the fibrin structure, remodelling and reabsorption within the first week of the healing process, and affect cell adhesion, migration and proliferation. Hyaluronic acid binds specifically to fibrinogen and increases the final

turbidity of fibrin *in vitro* by stimulating the polymerisation process. Hyaluronic acid-containing fibrin gel supported migration of a variety of cells types including EC, but not capillary morphogenesis (Nehls and Drenckhahn 1995). Capillary morphogenesis depended on gel configuration, and was preferably stimulated on malleable gels, whereas rigid gels supported cell migration, and inhibited capillary morphogenesis (Nehls and Herrman 1996). Moreover, the polymerization of fibrin was influenced by a number of plasma proteins, such as antithrombin III, fibronectin, albumin, γ-globulin, and plasminogen (Nehls and Herrmann 1996).

The concentration of von Willebrand factor, a marker of EC maturation, was higher in EC on all fibrin samples compared to PS. The highest concentration was found in EC on thick surface fibrin gels, and bulk gel. In our experiment, the concentration of von Willebrand correlated proportionally with the concentration of $\alpha_v\beta_3$ integrin receptor in EC. A higher concentration of $\alpha_v\beta_3$ integrin receptor was observed on thick fibrin assemblies that resemble a more natural fibrin clot. This is in contrast with other studies, where $\alpha_v\beta_3$ expression was increased during capillary sprouting, angiogenesis and granulation tissue formation, but was decreased in mature blood vessels (Clark *et al.* 1996).

The poor mechanical properties of fibrin gel limit its use in tissue engineering. However, they can be greatly improved in bioreactor systems. Fibrin gel moulded to the shape of a heart valve was seeded with a mixture of VSMC and fibroblasts, and subjected to perfusion in a special heart-like bioreactor. After 12 days, the fibrin constructs contained elongated cells incorporated in the fibrin gel matrix, and cells aligned along the vessel and the valve leaflet. In the valve constructs, both type I and type III collagens, fibronectin, laminin as well as chondroitine sulfate were present. Moreover, the fibrin was strong enough to manipulate without tearing (Flanagan *et al*. 2007). Fibrin beads seeded with periosteal cells were successfully used for the treatment of metadiaphyseal ulna defects in rabbits (Perka *et al*. 2000).

An important feature of fibrin is that it can be derived in a relatively high quantity from the patient's own blood and used in an autologous form for modifying biomaterials. Our subsequent study on fibrin assemblies coated with fibronectin, laminin, or collagen proved that coating with ECM proteins improved VSMC adhesion, spreading, and growth compared to polystyrene (Filová *et al*. 2005).

Natural ECM proteins contain cell adhesion ligands in the conformation that is necessary to make them accessible to cell adhesion receptors and for subsequent cell attachment and spreading. Fibrin layers coated with type I collagen, fibronectin, or laminin significantly

increased the initial attachment of VSMC compared to polystyrene, but only a slight rise in initial cell attachment was observed on pure fibrin. In addition, all protein assemblies improved the spreading of VSMC in comparison with polystyrene on day 1 after seeding. These results confirmed previous observations that fibronectin-coated PTFE vascular grafts enhanced endothelialisation compared to non-coated grafts (Seeger and Klingman 1988). In another study, purified fibrin *in vitro* was not able to induce angiogenesis. Angiogenesis was, however, stimulated after fibrin supplementation with VEGF or bFGF (Clark 2001).

In our study mentioned above (Filová *et al*. 2005), laminin decreased DNA synthesis of VSMC on day 3 after seeding, but did not influence cell proliferation after 7 days compared to polystyrene. This is in agreement with the study performed on airway smooth muscle cells, where laminin decreased their proliferation and increased their synthesis of contractile proteins (Hirst *et al*. 2000). Similarly, on a layer composed of laminin plus type IV collagen, aortic VSMC cells produced a higher level of α-actin compared to fibronectin (Thyberg and Hultgårdh-Nillsson 1994). We observed increased VSMC proliferation on pure fibrin, fibrin coated with either fibronectin or type I collagen. Two-dimensional layers of both collagen I and fibronectin were observed to support VSMC in shifting towards their synthetic state, whereas three-dimensional matrices of the same proteins maintain their contractile phenotype (Yamamoto *et al*. 1993; Stegemann and Nerem 2003). Fibronectin embedded fibrin gel supported capillary formation of EC, and inhibited their migration (Nehls and Drenckhahn 1995). Interestingly, polymerised collagen fibrils have been observed to decrease thymidin incorporation into the DNA of smooth muscle cells more than 10-fold compared to monomer collagen. The reduction of cell proliferation was caused by up-regulation of Cdk2 inhibitors $p21^{Cip/Waf1}$ and $p27^{Kip}$ (Koyama *et al.* 1996).

VSMC seeded on a textured polydimetylsiloxane tissue scaffold were able to remodel fibronectin into fibril-likes structures oriented parallel to the grooves. On nontextured substrata, no fibronectin fibrils were found (Sarkar *et al*. 2005). Fibrilar fibronectin has been found to play an important role in the assembly of fibrillar collagen, and subsequently in the mechanical properties of ECM (Li *et al*. 2003). Both type I and type III collagens contribute to the stability of the vessel wall, as mutations in their molecules lead to blood vessel rupture (Sottile 2004).

Cells on all fibrin samples contained dot-like focal adhesion plaques, but the most distinct adhesions were found on pure fibrin and on fibrin either coated or mixed with type I collagen. On polymerized type I collagen, but not on monomeric collagen, decreased

formation of both focal adhesion sites (stained for vinculin) and stress fibres were observed (Koyama *et al*. 1996).

In addition to the studies performed on entire fibrin or ECM molecules, our further studies were focused on biomimetic surfaces functionalized with synthetic oligopeptidic ligands for cell adhesion receptors derived from these molecules. The creation of these surfaces started by depositing poly(L-lactide) (PLLA) on microscopic glass coverslips. PLLA was chosen because it could act as a suitable bulk material for a newly constructed bioartificial tissue, e.g. a vascular wall. The PLLA films were then modified with pure poly(DL-lactide) (PDLLA) or PDLLA and PDLLA-*b*-PEO block copolymers, in which either none, 5% or 20% of the copolymer molecules carried a synthetic fibronectin-derived ligand for integrin adhesion receptors, the GRGDSG oligopeptide, attached to the end of the PEO chain (Bačáková *et al*. 2007).

In the case of pure non-functionalized PDLLA-*b*-PEO, the PEO on the surface of the material bore only the inert methoxy group. In addition, this material was highly hydrophilic and did not support cell adhesion and growth. However, when the PEO chains were bound with RGD, the material surfaces became biomimetic, i.e., they were able to restore the adhesion and growth of VSMC in an extent similar to or only slightly lower than on the native ECM proteins. In additon, the cells adhered and grew in both serum-supplemented and serum-free media, where the potential influence of cell adhesion-mediating ECM molecules, mainly vitronectin and fibronectin, is minimized. Therefore, these results suggest that the integrin receptors on VSMC were bound specifically to the GRGDS sequences on PDLLA-*b*-PEO but not to the serum-derived molecules, which have been reported to have some potential to adsorb on to RGD-functionalized PEO surfaces (for a review, see Bacakova *et al.* 2007).

In our study, hydrophilic PEO was used to create an inert background that bore the functional biomimetic oligopeptides. Cells growing on the pure PDLLA-b-PEO copolymer were round, clustered in aggregates, and without focal adhesions. Surface wettability, chemical composition, and electrical charge influence the amount and spatial conformation of proteins that adsorb on to a biomaterial surface. Extremely hydrophilic PEO surfaces have been reported to inhibit protein adsorption due to their repulsive properties (Bosker *et al*. 2005). Higher adsorption of collagen was observed on hydrophobic polystyrene than on more hydrophilic plasma-oxidized polystyrene. However, on moderately hydrophilic surfaces created by plasma treatment, the collagen layers were smoother and showed higher mechanical stability (Pamuła *et al*. 2004). Fibronectin was adsorbed in an active conformation only on moderately hydrophilic surfaces (Dewez *et al*. 1999). We observed better

antiadhesive properties in PDLLA-b-PEO copolymers with a longer PEO chain (Mw = 10,000-20,000) compared to surfaces with shorter PEO chains of Mw = 6,000 (Filova *et al*. 2006). Other studies have also confirmed that a longer chain and higher grafting densities improved its resistance to protein adsorption (Griffith 2000).

During cell adhesion, cells bind to specific amino sequences, e.g. Arg-Gly-Asp present in ECM proteins, such as vitronectin, fibronectin, collagen, laminin, and osteopontin by integrin receptors (Salasznyk *et al*. 2004). The spontaneous adsorption of proteins from a cell culture medium or blood on to the biomaterial surface is rather random, i.e., hardly controllable and less defined, which in turn hampers precise control of cell adhesion. Chemically well-defined surface-attached oligopeptides provide control over the density and distribution of the cell-material contacts, and consequently over cell behaviour. In our study, an RGD sequence was used in the form of a longer oligopeptide, i.e. GRGDSG, in order to increase both the chain flexibility and accessibility for cell integrin receptors. We observed significantly improved VSMC spreading on a surface containing 5% GRGDSG compared to 20% GRGDSG and a PEO surface without ligands in serum-supplemented media (Bačáková *et al*. 2007). Similarly, in other studies, the RGD oligopeptide allowed cell attachment and spreading in a wide range of spacing from 10 nm to 440 nm (Massia and Hubbell 1991; Humphries *et al*. 1986). This may be due to the altered three-dimensional conformation of oligopeptide at higher concentrations, which can make ligands inaccessible for cells. A GRGDSG-PEO-b-PDLLA surface markedly restored the formation of vinculin-containing focal adhesion plaques at a concentration of 5 %, though the focal adhesions were smaller, less numerous, and were located preferably at the cell periphery. In serum-free media, cells were not viable on both PDLLA-b-PEO and PDLLA without GRGDS during 3-day cultivation. However, the positive effect of GRGDSG on VSMC spreading, proliferation, and focal adhesion formation was less pronounced in a serum-free medium compared to a serum-supplemented medium. This may be due to the lack of a cooperative sequence, e.g. PHSRN present in fibronectin, that stabilises the conformation of both ligand and integrin receptors (Benoit and Anseth 2005). Another explanation is a lower concentration of growth factors in the serum-free medium, because their only source was autocrine production by VSMC colonizing the material.

Our studies on three-dimensional PLGA matrices demonstrate that the growth of osteoblast-like MG-63 cells in PLGA matrices depends on the properties of the scaffold, especially pore size. Our first set of porous PLGA scaffolds was prepared by a standard solvent casting/particulate leaching technique (Pamuła *et al.* 2008a). During this procedure, the sedimentation of porogen, i.e. sodium citrate particles, during slow solvent evaporation

from the material in Petri dishes causes the formation of a thin nonporous polymer layer, i.e. a polymeric crust, in the surface layer of the samples. Subsequently, the pores were opened by grinding the scaffolds. To prevent crust formation and to ensure that the pores opened, in the following experiments, individual PLGA samples were cut from a bigger cylindrical scaffold (Pamuła *et al*. 2008b). In addition to the standard procedure performed in the first experiment, the samples were perfused with solvent vapors to increase the interconnectivity of the pores.

Regardless of how the samples were prepared, we obtained similar scaffold porosities in the range of 83-88%, which is in agreement with other authors (Chen *el al*. 2002; Zhao *et al*. 2002). Higher porosity was found to slow down the degradation of PLGA scaffolds (Wu and Ding 2005). However, bigger pore size at the same porosity resulted in a high degradation rate. Compressive yielding of PLGA scaffolds was observed after 16 weeks for 50-90 μm pores, after 11 weeks for 90-180 μm pores and 180-280 μm pores, and after 8 weeks for 280-450 μm pores (Wu and Ding 2005). This means that quite fast polymer degradation significantly reduces the time available for cell ingrowth and synthesis of a sufficient amount of ECM.

The permeability of our PLGA scaffolds, ranging from 17-94 μm^2, increased with increased pore size, but was not affected by the way in which the scaffold had been prepared. The permeability obtained in our experiments was similar to that of cancellous bone (1.5-140 μm^2) (Hui *et al*. 1996). As permeability denotes pore interconnectivity, it is a prerequisite for sufficient diffusion of nutrients, waste and material degradation products through the scaffold.

Pore size, pore distribution, pore interconnectivity, and scaffold porosity strongly affect the osteoconductivity of the bone graft as well as its biocompatibility. In our experiments, the highest viability of MG-63 cells (measured as an activity of mitochondrial enzymes) was observed on PLGA scaffolds with pores bigger than 200 μm in diameter, and increased DNA synthesis on scaffolds with the biggest, i.e. 600 μm, pores. A pore size of 40μm seems to be unsuitable for bone repair, as MG-63 cells did not considerably penetrate into the pores, but they spanned over the pores (Pamuła *et al.* 2008a). Similarly, microvascular epithelial cells created a multilayer on the surface of PLA scaffolds, and did not penetrate into the pores when the pore diameter was smaller than 38 μm (Zeltinger *et al*. 2001). To enable osteoblast penetration, the pores should be at least 100-150 μm in diameter (Simon *et al*. 2003; Hulbert *et al*. 1970), though the optimal size of pores for bone tissue engineering is thought to be in the range of 200-800 μm (Gugala and Gogolewski 2005). Pores that are smaller than 100 μm are preferably filled with connective tissue. For rapid *in vivo* revascularization of the graft, the suggested pore size is greater than 500 μm (Wake *et al*. 1994). In our experiments, both

proliferation and penetration of MG-63 cells into the pores increased with pore size. On scaffolds with pores above 200 μm, MG-63 cells were found throughout the volume of the scaffolds, though most of them penetrated to a maximum depth of 410-800 μm. Rat calvarial osteoblast cultured on PGLA foams with pore diameters in the range of 150-300 and 500-710 μm achieved similar densities on all samples, and the cells penetrated to a depth of 190-220 μm after 56-day cultivation in a culture (Ishaug-Riley *et al*. 1998). After 11-week implantation, calcified tissue was observed at a maximum depth of 300 μm in ceramic pellets with pores of 100-150 μm (Hulbert *et al*. 1970). Among PDLLA scaffolds with pores of 75, 250, 400, and 750 μm implanted in rabbits for 12 weeks, the most effective regeneration was observed on the scaffold with a pore size of 250 μm. Sponges with 75 μm pores were filled with connective tissue (Gugala and Gogolewski 2005).

In both *in vivo* and *in vitro* experiments, maximum cell densities are usually observed in the margins of the scaffold and low cell densities are observed in the centre of the scaffold. A simple one-dimensional model of glucose diffusion within porous scaffolds showed a drop in glucose concentration beyond a few hundred microns in the absence of flow (Botchwey *et al.* 2003). In organisms, both nutrition limitations and cell hypoxia appear at a distance greater than 200 μm from a blood vessel (Hsiong and Mooney, 2006). Limited diffusion causes an inadequate supply of nutrients and oxygen, and accumulation of waste and degradation products. To improve diffusion through the samples, scaffolds with bimodal distributions of the pores were designed. Bigger pores serve as a space for cell ingrowth and ECM deposition, while small pores (<10 μm) should ensure appropriate diffusion of nutrients and metabolites. In addition to nutrition supply, the faster diffusion of PLGA degradation products prevents their accumulation and provides a gradual release without a massive burst at the final stage of degradation (Sosnowski *et al*. 2006). PLLA/PLGA scaffolds that contained both micropores in the range from 50 to 400 μm and mesopores in the range from 2 nm to 5 μm supported cell viability compared to standard microporous samples without mesopores, and allowed homogeneous cell distribution throughout the sample volume after 34 days of culture (Sosnowski *et al*. 2006). Similarly, the homogeneity of cell growth throughout the scaffold volume can be increased by increasing the size of the pores up to 1 mm (Simon *et al*. 2003), or by using centrifugal forces during cell seeding. This is effective mainly if low cell seeding densities are used (Godbey *et al*. 2004). Rapid prototyping technology known as fused deposition modelling is a computer-controlled scaffold preparation method that assured precise control of scaffold porosity, pore volume and structure, and allowed continuous cell distribution throughout the scaffold thickness (Cao *et al*. 2003). Various dynamic cultivation

systems, such as rotating bioreactors and perfusion systems, have been developed to preserve proper perfusion of the scaffolds (Botchwey *et al.* 2003). However, long-term cultivation of osteoblast-seeded PLGA scaffolds in a spinner flask bioreactor was accompanied by a marked decrease in cell density inside the scaffold, possibly due to cell death (Shea *et al*. 2000).

Both two- and three-dimensional poly(L/DL-lactide) were observed to have an osteoconductive potential (Gugala and Gogolewski 2005; Karp *et al*. 2003). On PLGA scaffolds of different pore sizes, the alkaline phosphatase activity of stromal osteoblastic cells was as high as on tissue culture polysterene (Ishaug *et al*. 1997). Similarly, in our experiment, the concentration of both osteocalcin and osteopontin (i.e., calcium-binding ECM glycoproteins and important markers of osteogenic cell differentiation) in MG-63 cells were the same on scaffolds with pores of 180-200 μm, 250-320 μm, and 400-600 μm, PGLA foil and polystyrene. On the other hand, rat calvarial osteoblasts expressed higher alkaline phosphatase activity when cultured on PLGA scaffolds with a pore size of 500-710 μm compared to scaffolds with a pore size of 150-300 μm 7 days after seeding. After 14 days in a culture, mineral deposits began to appear on the top of the constructs (Ishaug-Riley *et al*. 1998). Simultaneously, it was observed that rat calvarial osteoblasts seeded at a lower seeding density of $11.1x10^5$ cells per cm^2 had higher alkaline phosphatase activity than cells seeded at a density of $22.1x10^5$ cells/cm^2 on scaffolds with a pore size of 150-300 μm (Ishaug-Riley *et al*. 1998). Both osteogenic differentiation and calcium deposition of neonatal rat calvarial osteoblasts were further enhanced on a PLGA/hydroxyapatite scaffold (Kim *et al*. 2006).

Our other studies in the field of cell-biomaterial interaction have focused on micropatterned surfaces, i.e. surfaces with domains adhesive and non-adhesive for cells. Micropatterned surfaces were prepared with a strip width ranging from 20 to 500 μm (Thakar *et al*. 2003; Barbucci *et al*. 2003). In our experiments, we cultured VSMC, EC, mesenchymal stem cells and skeletal muscle cells on surfaces patterned with strips made of acrylic acid or 1,7-octadiene that were 75 and 150 μm in width (Filova *et al*. 2008b). We observed preferential adhesion and growth of all studied types of cells on a hydrophilic acrylic acid strip. This regional selectivity in cell adhesion was most pronounced in experiments with mesenchymal stem cells, VSMC and EC, and less pronounced in skeletal muscle cells. In addition, we observed a different behaviour of various cell types on acrylic acid microdomains compared to continuous non-patterned acrylic acid surfaces. In both EC and mesenchymal stem cells, the growth on acrylic acid domains was increased, whereas in VSMC this growth was similar, and in skeletal muscle cells, it was even slower than on the continuous surfaces.

On acrylic acid domains, VSMC were elongated, and were markedly stained for alpha-actin, a marker of VSMC differentiation. In a similar study, VSMC growing on 20- and 30-μm wide collagen strips decreased both the formation and the content of alpha-actin compared to 50 μm-wide strips, although the cells were aligned on all surfaces (Thakar *et al*. 2003). Moreover, on narrow, i.e. 20- and 30-μm strips, lower BrdU incorporation in VSMC was found compared to 50-μm wide strips. In our experiments, no difference in BrdU incorporation of VSMC was found between patterned and unpatterned surfaces. Interestingly, VSMC growing on a textured polydimethylsiloxane surface were able to reorganize fibronectin into fibres parallel to the grooves, while no fibronectin fibres were observed on smooth surfaces (Sarkar *et al*. 2005).

The size of squared fibronectin domains on antiadhesive fluid lipid bilayers, ranging from 5 to 40 μm, regulated the spreading and morphology of EC (Kam and Boxer 2001). In addition, EC were able to bridge over narrow antiadhesive lipid regions and span over multiple fibronectin domains. The same cell bridging over 1,7-octadiene strips was also observed in our study (Filova *et al*. 2008b). In EC growing on domains composed of acrylic acid, significantly higher BrdU incorporation was observed in comparison with both antiadhesive domains made of 1,7-octadiene and unpatterned acrylic acid surfaces. Moreover, EC growing on acrylic acid strips were stained intensively for von Willebrand factor, suggesting their better maturation. On micropatterned collagen, increased migration of EC subjected to shear stress was observed in the flow direction, which can promote wound healing, angiogenesis, and vascularisation of engineered tissues (Hsu *et al.* 2005).

Cell response to flat micropatterned surfaces with adhesive and antiadhesive domains was found to be stronger with decreased periodicity of the surface. The cell behavior on micropatterned surfaces also depended on the cell type, and on its ability to bridge non-adhesive regions. In addition, primary cell cultures were found to be more sensitive to surface patterning than transformed cell lines (Clark 1994; Kam and Boxer 2001).

In our experiments, we also found that hydrophobic 1,7-octadiene, used for constructing non-adhesive domains, was not entirely repulsive for cells. This can be explained by the fact that both laminin and collagen (Clark 1994; Filova *et al*. 2008b; Pamula *et al.* 2004; Dewez *et al*. 1999) are preferentially adsorbed on hydrophobic surfaces. On the other, hand fibronectin is adsorbed in its active conformation only on hydrophilic surfaces (Clark 1994).

6. CONCLUSIONS

All materials developed and investigated in this thesis gave good supports for the adhesion, growth, viability, metabolic activity and maturation of vascular and bone-derived cells.

Fibrin layers were developed for potential coating of biomaterials and for enhancing their bioactivity. These layers were arranged two- or three-dimensionally and were of various thicknesses. Two-dimensional ultrathin fibrin layers were prepared using PPACK and hirudin, which are capable of inhibiting thrombin bound to fibrin. We found that these two-dimensional fibrin layers supported EC spreading and proliferation. These ultrathin fibrin layers can potentially be used for coating pore walls inside a three-dimensional scaffold to enhance cell attachment. Three-dimensional thin surface fibrin assemblies were obtained using AT III and heparin, which inhibit thrombin in solution. Three-dimensional thick surface fibrin gels were obtained using a non-inhibited fibrinogen solution. EC growing on thick fibrin gels contained a higher concentration of von Willebrand factor, indicating a high maturation status of these cells. Three-dimensional thick bulk fibrin gels, similar to a natural fibrin clot, seemed to be best for proliferation and differentiation of EC.

Fibrin layers modified with ECM proteins, such as type I collagen, fibronectin and laminin, also had supportive effects on cell colonization. On these layers, increased initial attachment, spreading and growth of VSMC compared to pristine polystyrene were observed. Pure fibrin and fibrin covered or mixed with collagen improved mainly VSMC adhesion, while fibronectin-coated fibrin supported their proliferation. Both pure and protein-coated fibrin assemblies can be used to modify the inner surface of bioartificial vascular prostheses.

To avoid the use of allogeneic or xenogeneic proteins in tissue engineering, and to gain more precise control over cell adhesion, synthetic well-defined polymers functionalized with short oligopeptides, i.e. ligands for integrin adhesion receptors, were developed. The functionalized surfaces were made of PDLLA-b-PEO grafted with 5% or 10% GRGDSG oligopeptide. We found that antiadhesive non-grafted PDLLA-b-PEO copolymer did not allow attachment and growth of VSMC. On a PDLLA-b-PEO-GRGDSG surface, however, cells were able to attach, spread, form focal adhesion plaques, synthesize DNA, and proliferate in both serum-supplemented and serum-free media.

Three-dimensional scaffolds are required for bone repair, and serve as biodegradable templates for ingrowth and regeneration of new bone tissue. We prepared PLGA scaffolds with different pore sizes using a solvent casting/particulate leaching technique. Increased pore

size improved cell penetration and proliferation, though it did not affect their differentiation. Human osteoblast-like MG-63 cells grew mainly in the upper part of the scaffolds. Homogeneity of cell distribution throughout the scaffold can be improved by cultivation in perfusion or rotating culture systems, or *in vivo*.

Micropatterned surfaces are a suitable approach for directing the growth, morphology and spatial distribution of cells over the surface of the material. In this study, surfaces patterned with hydrophilic and hydrophobic microdomains were prepared by plasma polymerisation of acrylic acid and 1,7-octadiene, respectively, and seeded with vascular smooth muscle cells, endothelial cells, human skeletal muscle cells and mesenchymal stem cells. All cell types grew preferentially on hydrophilic acrylic acid domains. The most selective growth on these domains was observed in mesenchymal stem cells. Moreover, these cells contained a higher concentration of beta-actin, while on hydrophobic and less convenient 1,7-octadiene they synthesized more CD44, a receptor for hyaluronic acid. In VSMC growing on acrylic acid domains, a higher amount of alpha-actin was found, indicating their better differentiation. Similarly, EC on these domains developed numerous Weibel-Palade bodies brightly stained for von Willebrand factor, i.e. an important marker of EC maturation.

7. SUMMARY

Degradable or non-degradable polymers are widely used for constructing artificial vascular or bone prostheses; however, it is necessary to improve the properties of the prostheses in order to reduce their thrombogenicity and increase their biocompatibility, bioactivity and mechanical stability.

The aims of the study were to prepare and evaluate materials made of biopolymers, namely fibrin and ECM proteins, or synthetic polymers, such as copolymers of PDLLA and PEO functionalized with GRGDSG oligopeptides (i.e., ligands for integrin adhesion receptors on cells), PLGA scaffolds and micropatterned surfaces made of hydrophilic acrylic acid and hydrophobic 1,7-octadiene. All these materials were evaluated with regard to their effects on cell attachment, growth and differentiation, as well as their potential use in medicine.

As for the biopolymers, we prepared fibrin assemblies of various thicknesses and spatial arrangements, i.e. in the form of two- and three-dimensional layers or three-dimensional bulk gels, and the assemblies were seeded with EC. We found that a two-dimensional fibrin layer supported EC spreading and proliferation, but that EC contained a lower concentration of von Willebrand factor, a marker of their differentiation, compared to three-dimensional thick bulk gels.

Fibrin assemblies coated with ECM matrix proteins, i.e. type I collagen, fibronectin, or laminin, increased the initial attachment, spreading and growth of VSMC compared to pristine polystyrene. Pure fibrin and fibrin covered or mixed with collagen improved mainly cell adhesion, while fibronectin-coated fibrin distinctly increased the proliferation of VSMC.

The functionalized surfaces were made of PDLLA-*b*-PEO copolymers grafted with 5% or 10% GRGDSG oligopeptide. We found that the non-grafted PDLLA-*b*-PEO copolymer was antiadhesive, i.e., it did not allow attachment and growth of VSMC. On a PDLLA-b-PEO-GRGDSG surface, however, cells were able to attach, synthesize DNA, and proliferate even in a serum-free medium, which suggests a specific binding between the GRGDSG sequences and the integrin adhesion receptors on the cells.

PLGA scaffolds with different pore sizes were able to control cell behaviour in static cultures. Increased pore size improved the penetration and proliferation of human osteoblast-like MG-63 cells, though their differentiation, measured by the concentration of osteocalcin, was not affected. In addition, the static culture system was not able to ensure homogeneity of the cell distribution throughout the scaffold volume.

Micropatterned surfaces prepared by plasma polymerisation of hydrophilic acrylic acid and hydrophobic 1,7-octadiene were able to guide the adhesion and growth of vascular smooth muscle cells, endothelial cells, human skeletal muscle cells and mesenchymal stem cells. Among the four cell types, mesenchymal stem cells grew most selectively on hydrophilic acrylic acid microdomains. On these domains, mesenchymal stem cells contained a higher concentration of beta-actin, while on hydrophobic and less convenient 1,7-octadiene they synthesized more CD44, a hyaluronan receptor involved in cell-cell adhesion. In VSMC growing on acrylic acid domains, a higher amount of alpha-actin was found, which indicated their higher differentiation status.

Both natural and synthetic polymers can be used to produce or modify vascular and bone prostheses. The polymers tested in the study were able to improve cell attachment, growth and differentiation, which are necessary prerequisites for obtaining a biocompatible and patent graft, and also to improve the three-dimensional structures that enable bone tissue ingrowth and regeneration.

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FILOVÁ E., BAČÁKOVÁ L., CHLUPÁČ J., HOUSKA M., RIEDEL T., LISÁ V., BRYNDA E.: Growth and Differentiation of Endothelial Cells on Fibrin Assemblies Modified with Extra-cellular Matrix Proteins

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Poster at the 5th Tissue Engineering Symposium, April 23-25, 2008, Tampere, Finland

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Poster at the 8th World Biomaterials Congress, May 28-June 1, 2008, Amsterdam, Netherlands

11. SCHOLARSHIPS

30.6.-13.6.2004: AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Krakow, Poland (Prof. Stanislaw Blazewicz)

16.7.-13.8.2004: Short term scientific mission (organized by COST): Department of Engineering Materials, University of Sheffield, Sheffield, UK, (Dr. Alex Shard)

10.2.-18.2.2005: 3D-cultivation Workshop, Institute of Molecular Biology, Homburg, Saar Germany (Prof. Gerhard Unteregger)

3.4.-16.4.2005: AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Krakow, Poland (Prof. Stanislaw Blazewicz, Dr. Elzbieta Pamula)

14.7.-20.7.2007: Summer School on Nanosciences & Nanotechnologies, Laboratory for Thin Films - Nanosystems & Nanometrology (LTFN) Aristotle University of Thessaloniki, Physics Department 54124, Thessaloniki, Greece (Dr. Stergios Logothetidis, Professor at the Physics Department of Aristotle University of Thessaloniki).

The prize for the best presentation – during the N&N07, GREECE conference and 4th International Workshop on Nanosciences & Nanotechnologies - NN07, July 16-18, 2007, Thessaloniki, Greece.

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