Adhesion, growth and differentiation of osteoblasts on surface-modified materials developed for bone implants

Marta Vandrovcová, Lucie Bačáková

Department of Growth and Differentiation of Cell Populations, Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4 – Krč

Corresponding Author:

Lucie Bačáková, MD, PhD

Dept. of Growth and Differentiation of Cell Populations

Institute of Physiology

Academy of Sciences of the Czech Republic

Videnska 1083

142 20 Prague 4 - Krc

Czech Republic

Phone: +420 2 9644 3743, +420 2 9644 3742

Fax: +420 2 9644 2488, +420 2 4106 2488

E-mail: lucy@biomed.cas.cz

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Summary

This review briefly outlines the history and possibilities of bone reconstruction using various types of artificial materials, which allow interaction with cells only on the surface of the implant or enable ingrowth of cells inside the material. Information is also provided on the most important properties of bone cells taking part in bone tissue development, and on diseases and regeneration. The most common cell types used for testing cell-material interaction *in vitro* are listed, and the most commonly used approaches to this testing are also mentioned. A considerable part of this review is dedicated to the physical and chemical properties of the material surface, which are decisive for the cell-material interaction, and also to modifications to the surface of the material aimed at integrating it better with the surrounding bone tissue. Special attention is paid to the effects of nanoscale and microscale surface roughness on cell behaviour, to material surface patterning, which allows regionally-selective adhesion and growth of cells, and also to the surface chemistry. In addition, coating the materials with bioactive layers is examined, particularly those created by deposition of fullerenes, hybrid metal-fullerene composites, carbon nanotubes, nanocrystalline diamond films, diamond-like carbon, and nanocomposite hydrocarbon plasma polymer films enriched with metals.

Key Words: surface roughness and topography, surface wettability, surface coating, surface patterning, osteogenic cells, bone tissue engineering

1. Introduction

The lifestyle and attitudes of people nowadays are responsible for the more and more frequent manifestation of so-called “diseases of civilization”. The most common diseases include heart and vascular diseases, cancer, obesity, diabetes, inflammatory rheumatic joint

diseases, premature birth or miscarriage, depression, etc. The causes of these diseases are various and can be combined. They comprise mainly the intake of high-calorie foods, high consumption of animal products, decreased physical exertion, excessive alcohol drinking and cigarette smoking, and mental stress. Humans usually have difficulty in adapting to the excessive amount of conveniences brought to us by civilization, in the best sense of this word. The food industry produces fatty, salty meals and sugary drinks. The development of transport has caused a decline in physical movement, and also contributes to injuries due to accidents. The lifestyle of "modern" humans paradoxically reduces their quality of life. Nevertheless, the human life span is constantly lengthening, and thus there is a need to extend people's productive life. Modern medicine has come on to the scene, and has been developing new medicaments and new therapeutic procedures, including the creation of tissue and organ replacements by tissue engineering methods. At the same time, however, disease prevention remains of great importance.

This review concentrates on ways to replace irreversibly damaged bone and joint tissue, especially by surface-modified materials that are highly attractive for adhesion, growth and osteogenic differentiation of cells, and thus supportive for integration of the implant with the bone tissue and its secondary stability. Prior to the construction of such implants, it is necessary to know about the physiology and pathophysiology of the bone tissue in health and disease.

Natural bone is composed of several types of cells of mesenchymal origin, such as osteoblasts, osteoclasts, stem cells and vascular cells. Osteoblasts are responsible for bone growth, while osteoclasts are responsible for bone resorption. Bone is resorbed and replaced in a physiological process referred to as bone remodelling (Barrett *et al.* 2010). Osteoblasts are very similar to fibroblasts; all the genes expressed in fibroblasts are also expressed in osteoblasts. The only difference is in the expression of two osteoblast-specific transcripts: one

encoding Cbfa1, and the other encoding osteocalcin. Cbfa1 is an osteoblast-specific transcription factor, considered as the earliest and most specific marker of osteogenesis. Osteocalcin is the most abundant non-collagenous protein of the bone matrix. This molecule inhibits bone growth by inhibiting the activity of transglutaminase, binds calcium and is also an important marker of osteoblast differentiation (Kaartinen *et al*. 1997, Ducy *et al.* 2000). During embryonic development, osteoblast differentiation can occur through two distinct pathways, i.e. intramembranous and endochondral ossification. Intramembranous ossification mainly occurs during formation of the flat bones of the skull. In this case, the mesenchymal progenitor cells differentiate directly into osteoblasts. Endochondral ossification, on the other hand, occurs in long bones, such as limbs. Long bones are formed from cartilage. Upon vascular invasion into the cartilage template, the chondrocytes die through apoptosis, are replaced by osteoblasts, and the template is transformed into bone by the ossification process (Barrett *et al.* 2010).

In healthy bone, there is a delicate balance between the synthetic activity of osteoblasts and the resorptive activity of osteoclasts. For example, during ageing and in some kinds of diseases, the balance shifts in favour of osteoclasts, which can lead to increased bone resorption. This process affects the bone structure, and makes the bone brittle and susceptible to fractures. One of the most common bone diseases is osteoporosis. It is mostly caused by a decrease in estrogen production in women during menopause (Rodan and Martin 2000). Estrogen loss is associated with elevated bone resorption caused by a rise in osteoclast number, which is driven by an increase in the cytokine production that regulates osteoclast generation by the following cascade: receptor for the activator of nuclear factor-κB (RANK) ligand; tumor necrosis factor-α (TNF-α); interleukin-1, interleukin-6, interleukin-11; macrophage-colony stimulating factor (M-CSF); and prostaglandin E (Pacifici 1998).

Degeneration of the articular cartilage (by aging, wear, etc.), i.e. primary osteoarthritis, can be considered as the main cause of joint diseases. However, secondary osteoarthritis (caused e.g. by trauma or by disability) is also an important factor. Both primary and secondary osteoarthritis are manifested by pain during walking, which can be partially suppressed by palliative remedies, e.g. painkillers. Movement itself, however, may be a problem, even an impossibility. The only way to restore mobility is often by implanting an artificial joint (Sedlák and Píška 2008). The worldwide investment in bone implants was about 23 billion dollars in 2005, and this figure has been increasing year by year. Metal implants form the main category, though these materials are less suitable for bone tissue engineering.

2. Tissue engineering and artificial materials

Tissue engineering has been defined as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (Langer and Vacanti 1993). In order to aid tissue reparation or regeneration, tissue engineering can use purely biological building blocks, e.g. living cells and extracellular matrix (ECM) molecules, or cells on carriers made of artificial materials. Thus, interactions between biomaterials and cells play an important role in this field. It has been repeatedly shown that cell behaviour is strongly dependent on the physical and chemical properties of the surface of the material (Linez-Bataillon *et al*. 2002, Eisenbarth *et al.* 2002, Webster and Smith 2005).

Artificial materials have been used for hundreds years. The Mayans, for example, created teeth from sea shells in ~600 A.D. Similarly, an iron dental implant was found in Europe in a corpse dated from 200 A.D. However, biomaterials as we think of them today did not exist just 50 years ago. There were no manufacturers of medical devices (except glass eyes, dental

devices, some external prostheses, etc.), no understanding of biocompatibility, no legal regulations, etc. (Ratner *et al.* 2004).

Materials used nowadays can be divided into several basic groups: synthetic polymers, natural polymers, ceramics, metals, and composites which combine together the properties of two or more materials of different classes.

Each of the above-mentioned groups of materials can be used in bone tissue engineering. Generally, synthetic polymers are used for fabricating some orthopaedic and dental devices, and also for artificial tissue replacements. Poly(methyl methacrylate) is, for example, a main component of bone cement for orthopaedic surgery. Poly(dimethyl siloxane), well-known from its application in constructing artificial heart valves, is used in finger joint prostheses. Medical fibres, made e.g., of polytetrafluoroethylene (PTFE) or poly(lactide-co-glycolide) (PLGA), can be used as ligament and tendon substitutes (e.g. to fix separated shoulder joints or to repair knee joints). However, the application of polymers is limited due to relatively frequent problems with abrasion and wear, poor bone attachment, excessively high flexibility or low hardness. High hardness is characteristic for ceramic materials, but these materials are usually excessively brittle. Ceramic materials, e.g. hydroxyapatite (HAp) or tricalciumphosphate, can be used advantageously in combination with polymers, particularly in the form of bioactive and biodegradable micro- and nanoparticles (for a review, see Abramson *et al*. 2004, Vagaská *et al.* 2010). Biodegradable materials are important for temporary mechanically supportive healing appliances, such as sutures and bone fixation devices. These materials also have great potential for bone tissue engineering, because temporary biodegradable scaffolds are expected gradually to be replaced by newly formed, fully regenerated and functional bone tissue. Natural polymers are similar to and often identical with native macromolecules. These molecules are non-cytotoxic, because they are of the body origin, and are also better available for cells, e.g. for binding with cell adhesion

receptors. However, natural polymers are frequently immunogenic, and are also associated with a risk of pathogen transmission. Moreover, their structural conformation is more complex than in synthetic molecules, which can lead to worse technological manipulation. One of the most interesting molecules for bone tissue engineering is collagen. Collagen is a major protein of the ECM of various tissues; especially collagen I is predominant in the bone, tendon and skin. It has been found that, in combination with chondroitin 6-sulfate, collagen modifies the kinetics and the mechanism for healing full-thickness skin wounds in rodents. A positive influence of this combination on the bone tissue is proposed.

A final group of materials - metals - are the dominant materials used in present-day orthopaedics (for a review, see Abramson *et al*. 2004). Materials that are currently widely used for surgical implants include 316L stainless steel, cobalt-chromium alloys, and titanium and its alloys. Amongst the metallic materials available for implant applications, titanium is considered to be the best for bone implants, and is extensively used in biomedical applications. There are several reasons for choosing titanium and its alloys, namely mechanical properties, chemical stability, and last but not least the well-known biocompatibility of Ti. In comparison with other alloys, titanium-based materials have a low modulus of elasticity, varying from 110 to 55GPa, and approach the elasticity modulus of natural bone (30GPa). In comparison, 316L stainless steel and chromium-cobalt alloys have a much higher modulus of elasticity (210GPa, 240GPa, respectively), and this can lead to prosthesis failure through loosening or fracture. Thus, the modulus of elasticity of an artificial bone implant should be as similar as possible to that of the bone (Geetha *et al*. 2009). As for the chemical properties of metallic implants, local adverse tissue reactions or allergic reactions caused by these implants originate from the release of metal ions from the implant (Long and Rack 1998). Titanium is a reactive metal. This means that in air, water or any other electrolyte, an oxide is spontaneously formed on the surface of the metal. This oxide is about

4 nm in thickness. The specific mode of oxide growth on titanium has the positive effect that no metal ion will reach the surface. Titanium oxide is a good insulator and can occlude anionic impurities such as chlorine and fluorine of phosphates. By contrast, conductible materials imply redox processes which lead to denaturation of macromolecules (Steinemann 1998).

3. Cell models and culture conditions for studying cell-material interaction

Materials designed for bone implantation are tested *in vitro* on cell cultures and then *in vivo* on laboratory animals. Material tests under *in vitro* conditions usually start with the use of cell lines. These lines represent homogeneous, well-defined, relatively easily available and cultivable cell populations, which enable screening of multiple material samples and are capable of giving reproducible results. Animal cell lines often used for biomaterial testing include osteoblast-like UMR-106 cells, mouse bone marrow MBA-15 cells and mouse progenitor MC3T3-E1 cells. The last-mentioned are cells originally with a fibroblast-like phenotype, capable of differentiating into osteoblasts in appropriate culture environments (Kanazawa *et al*. 2007). Human bone-derived cell lines include osteoblast-like cells CPC-2, TE-85, MG-63, SaOS-2, U-2OS. Although these cell lines were derived from osteosarcoma, they generally retained the most important markers of osteogenic cell differentiation, such as the activity of alkaline phosphatase and production of osteocalcin (Zhao *et al*. 2007, Rudnik *et al*. 2008, Grausová *et al*. 2009a, Kalbáčová *et al*. 2009).

Selected interesting and promising results obtained on cell lines are then usually verified on primocultured and low-passaged cells derived from animal and human bones. These cells included differentiated osteoblasts, such as osteoblasts from neonatal rat calvaria (Webster *et al*. 2000a,b; de Oliveira and Nanci 2004, de Oliveira *et al*. 2007), human osteoblasts (hOB) obtained from surgical bone specimens (Anselme and Bigerelle 2005), and also non-

differentiated stem cells, such as mesenchymal stem cells derived from the bone marrow (MSCs; Filová *et al*. 2009a) or embryonic stem cells (Bedi *et al*. 2009). Primocultured and low-passaged animal and human cells can be purchased from specialized companies (Webster and Ejiofor 2004), which makes their availability and use in biomaterial testing easier.

For cell cultivation on the tested materials, classical static and more advanced dynamic cell culture systems can be used. In this system, the material samples are inserted into cultivation vessels (usually dishes). They are immersed in the cell culture medium and seeded with cells suspended in the medium. For cell seeding, a drop in the cell suspension in the culture medium can also be applied selectively to the surface of the material. The classical static system is suitable mainly for cells on planar ("two-dimensional") material samples, though these cells can also be exposed to dynamic conditions, such as fluid shear stress generated by a flow of cell culture media (Scaglione *et al.* 2008, Kokkinos *et al*. 2009, Tan *et al.* 2010), hydraulic pressure (Gardinier *et al*. 2009) or stretch strain if the cells are cultured on elastic deformable materials, such as silicone rubber (Kaspar *et al*. 2000). These types of mechanical stimulation of osteoblasts or other osteogenic cells (e.g. bone marrow stromal cells, osteoprogenitor MC3T3-E1 cells, human osteoblast-like MG 63 cells) led to reorganization of the focal adhesion plaques and cytoskeleton, increased cell stiffness and particularly led to more pronounced osteogenic cell differentiation, manifested by a higher expression of the main osteogenic transcription factors Cbfa1 and Osterix, extracellular matrix molecules collagen I, osteopontin, osteocalcin and bone sialoprotein, and higher alkaline phosphatase activity.

Dynamic cultivation is essential for cells grown on three-dimensional polymeric, ceramic or composite porous or fibrous scaffolds that are often used in bone tissue engineering. Dynamic cultivation, especially if coupled with dynamic cell seeding, facilitates the penetration of cells into the pores of the material and colonization of the deep parts of the

scaffolds with cells. This is almost impossible in the conventional static cell culture system. In addition, dynamic systems usually improve the perfusion of the scaffolds with cell culture media, and thus they improve the delivery of oxygen and nutrients to the cells and the removal of waste products of the cell metabolism and of degradation of the scaffold (for a review, see Pamula *et al*. 2008, 2009). The most frequently used dynamic systems are perfusion bioreactors (Olivier *et al.* 2007), horizontally or vertically rotating bioreactors (Belfiore *et al.* 2009, Meretoja *et al*. 2009) and bioreactors generating pressure (Mauney *et al.* 2004). Various types of mechanical stimulation can also be combined in a single bioreactor, for example cyclic hydraulic pressure and fluid shear stress (Gardinier *et al*. 2009), centrifugal-force-induced fluid pressure in a "rotating-cup" bioreactor and compression pressure (Belfiore *et al.* 2009) or perfusion-generated fluid shear stress and compression stress (Bölgen *et al*. 2008). Similarly as on planar surfaces, the cells in 3D materials cultured under dynamic conditions also exhibited enhanced proliferation, viability and osteogenic differentiation in comparison with cells in the conventional static cell culture system (Mauney *et al.* 2004, Olivier *et al.* 2007, Meretoja *et al*. 2009). Osteogenic cell differentiation can also be significantly enhanced by the use of special osteogenic media, i.e. supplemented with dexamethasone, ascorbic acid, vitamin D_3 and beta-glycerolphosphate (Mauney *et al.* 2004, Kokkinos *et al*. 2009).

4. Physicochemical properties of the material surface

When constructing artificial materials, it is desirable to create materials promoting the attachment, migration, proliferation, differentiation, long-term viability and proper functioning of the cells. The adhesion of cells to artificial materials and their further performance are mediated by ECM molecules, such as vitronectin, fibronectin, collagen, laminin and also fibrin, a molecule of provisional ECM taking part in wound healing and

often used for modifying the surface of a biomaterial (Brynda *et al*. 2005, Filová *et al.* 2009b). These molecules are spontaneously adsorbed on the surface from the biological fluids, such as cell culture media, blood or intercellular fluid, and specific active sites on these molecules are then recognized and bound by adhesion receptors on cells, mainly integrins. Thus, cell adhesion-mediating ECM molecules have to be adsorbed in an appropriate amount, spectrum, and particularly spatial conformation and flexibility in order to be accessible for cell adhesion receptors. The adsorption behaviour of proteins is markedly influenced by the physical and chemical properties of the material surface, such as its polarity, wettability, electrical charge and conductivity, roughness and topography, compliance and others (Engler *et al.* 2004, Bačáková *et al.* 2004**;** Bačáková and Švorčík 2008). Modification of the tissue response by the roughness and topography of the material is one of the most important criteria for the production of biomaterials, because these criteria are decisive for the cell-material interaction and for integration of the material with the surrounding tissue (Clark 1994, Ito 1999, He *et al.* 2008).

4.1. Surface roughness

Intensive research is being carried out on modifications of the material surface aimed at enhancing the attractiveness of the material for cells and thus optimizing the integration of the material with the surrounding tissue. One of these modifications is induction of the nanoscale roughness and topography of the material surface. Nanostructured surfaces, i.e. those containing irregularities less than 100 nm, are believed to mimic the nanoarchitecture of natural tissues, e.g. the size of some ECM molecules or irregularities on these molecules, such as folding, branching, etc., and also the size of the extracellular parts of cell adhesion receptors. On nanostructured surfaces, cell adhesion-mediating ECM molecules are adsorbed in advantageous, almost physiological geometrical conformations, and thus the specific

bioactive sites in these molecules, such as amino acid sequences like RGD or osteoblast-binding sequence KRSR, are well accessible to cell adhesion receptors. In addition, among all ECM proteins, nanostructured surfaces preferentially adsorb vitronectin (due to its relatively small, linear and non-complicated molecule), and this protein is preferentially recognized by osteoblasts over other cell types (Webster *et al*. 2000a,b; Price *et al*. 2004).

A wide range of studies have compared the effects of micro- and nanoscale material surface topography on various types of cells (Lincks *et al.* 1998, Bačáková *et al.* 2001, Zhao *et al.* 2007, Khang *et al.* 2008, Liu *et al*. 2008, Mendonça *et al.* 2008). Most of these researchers consider that nanoscale topography provides greater support for the proliferation of bone cells. This result has been explained by the advantageous interaction between nanosize irregularities on the material surface and adsorbed cell adhesion-mediating molecules, as mentioned above, and thus improved osteoblast adhesion and spreading, which is a prerequisite for their good subsequent proliferation activity (Webster *et al.* 2000a,b; Christenson *et al*. 2007, Khang *et al.* 2008, Liu *et al.* 2008). Microscale irregularities can hamper cell spreading (Fig. 1), and thus they may slow down cell proliferation (Bačáková *et al.* 2001, Starý *et al.* 2003a,b). In accordance with this, Rosa and Beloti (2003), who compared the influence of various submicron- and microscale roughnesses of titanium substrates on human bone marrow cell growth, found that cell adhesion and proliferation decreased with increasing material surface roughness. In this study, surface roughness was measured by the R_a parameter, defined as the departure of the roughness profile from the mean line, which was 0.24 μm, 0.69 μm, 0.80 μm and 1.90 μm. Similarly, human osteoblasts in cultures on titanium substrates of the mean roughness amplitude (parameter S_a) from 0.53 μm to 2.52 μm spread more intimately on surfaces with low roughness amplitude than on rougher surfaces (Anselme and Bigerelle 2005). On the other hand, cells on rougher Ti samples (R_a 0.80 μm and 1.90 μm) contained higher amounts of total protein and showed

higher activity of alkaline phosphatase, i.e. an enzyme participating in bone tissue mineralization and an important marker of osteogenic cell differentiation (Rosa and Beloti 2003). Therefore, microscale roughness can support osteogenic cell differentiation, although the initial cell responses, monitored by cell adhesion and proliferation, were not ideal.

Similar cell behaviour was observed on surfaces with various nanoscale roughnesses. In a study by Webster *et al.* (1999), performed on primary rat calvarial osteoblasts in cultures on nanophase titania (grain sizes from 20 to 56 nm) and alumina (grain sizes from 20 to 67 nm), the highest numbers of initially adhering cells were found on TiO_2 with grain size of 20-32 nm and Al_2O_3 with grains of 20-49 nm. These numbers were significantly higher than on conventional titania and alumina, which were markedly rougher (grain sizes of 2120 nm and 177 nm, respectively). These favourable results on nanostructured surfaces were explained by the relatively large material surface area that was available for cell adhesion on ceramics with optimal surface nanoroughness, and also the improved adsorption, configuration and bioactivity of proteins that mediate osteoblast adhesion (Webster *et al.* 1999).

In our studies performed on human osteoblast-like MG 63 cells in cultures on TiO_2 or glass, nanoroughness of R_a = 40 nm (i.e., a value close to the optimum surface roughness suggested by Webster *et al*. 1999) also induced a larger cell spreading area and significantly higher cell numbers on day 7 after seeding than was induced by higher surface roughness of 100 and 170 nm (Vandrovcová *et al.* 2010). Similar cell behaviour was observed on nanocrystalline diamond films (NCD). Among these films of root mean square (RMS) roughness of 20, 270 and 500 nm, surfaces with RMS of 20 nm provided the best support for the initial adhesion of human osteoblast-like SaOS cells (measured by cell number 1 hour after seeding), for metabolic activity of these cells (measured by the activity of cellular dehydrogenases 48 hours after seeding), and also for their osteogenic differentiation (measured by alkaline phosphatase activity and mineral deposition by cells 11 days after

seeding). These beneficial effects of a nano-rough coating with RMS of 20 nm were explained by its similarity to the topography of a real bone surface (Kalbáčová *et al.* 2009).

The supportive effect of nano-rough surfaces on osteogenic cell differentiation was also demonstrated when flat and nano-rough surfaces were compared. Although the osteoblast colony occupancy (i.e., the total surface area occupied by each cell colony) was larger on conventional flat borosilicate glass surfaces than on nanophase ceramics (represented by alumina, titania and HAp with grain sizes of 24-67 nm), the synthesis of alkaline phosphatase and the deposition of calcium-containing mineral were significantly higher in osteoblasts on nanophase ceramics after 21 and 28 days of cultivation (Webster *et al.* 2000b).

Similarly, de Oliviera and Nanci (2004) compared the osteogenic differentiation of neonatal rat calvarial osteoblasts in cultures on conventional unmodified Ti and TiAlV discs and discs with nanoscale irregularities induced chemically by etching in a solution of H_2SO_4 and H_2O_2. Osteoblasts grown on the nanotextured surfaces, which contained nanopits with a honeycomb-like appearance and were in the 10 nm range in diameter, increased their secretion of bone sialoprotein and osteopontin, i.e. non-collagenous proteins typical for the bone matrix. The activity of alkaline phosphatase and the formation of mineralized bone-like nodules was also higher in cells on etched surfaces than on non-etched surfaces (de Oliveira *et al*. 2007).

Therefore, it can be concluded that surfaces of roughness in tens of nanometers are preferred by bone cells for adhesion, growth, differentiation and phenotypic maturation, rather than flat surfaces and surfaces with submicron- or micro-scale roughness.

4.2. Surface topography and patterning

Not only the size of the surface irregularities, but also their shape and distribution on the surface of the material are important for the cell-material interaction. For example, structures

with different diameter (0.5 and 2 μm) and R_a parameter (0.2 and 0.4 μm) but with the same porous shape, which were created on titanium surfaces by anodic oxidation, increased the number of initially attached cells to similar values and induced similar morphological features in cells such as formation of filopodia, induced similar shape and distribution of vinculin-containing focal adhesion plaques, and induced a similar spatial arrangement of the actin cytoskeleton (Zhu *et al.* 2004).

The density of the irregularities on the surface of the material was crucial for the cell-material interaction. For example, Rice *et al.* (2003) deposited white-sulphate latex particles (shaped as hemispherical protrusions 110 nm in height) on titanium oxide surfaces in densities covering 3%, 19%, 30%, or 43% of the material surface. Increasing densities of nanoparticles decreased the spreading and proliferation of rat calvarial osteoblasts but increased the expression of osteocalcin in these cells. Similar results were obtained in experiments by Kunzler *et al.* (2007) performed on poly(ethylene imine)-coated silicon wafers adsorbed with silica nanoparticles (diameter 73 nm) in a gradient ranging from 0% to 21% coverage of the material surface. Rat calvarial osteoblasts in cultures on these surfaces exhibited a significant decrease in spreading, proliferation and formation of actin cytoskeleton, which was explained by more pronounced bending of the cell membrane, cell stress and reduced cell-material contact area. Thus, it can be concluded that increased density of surface irregularities had similar effects to increased size of these irregularities, as described above (paragraph 4.1.).

4.3. Surface chemical state of the material

Several studies have investigated whether surface roughness and topography, on the one hand, or surface chemistry, on the other, has a higher influence on cell behaviour on a material surface. Webster *et al.* (1999) stated that enhanced adhesion of rat calvarial

osteoblasts in cultures on nanophase alumina and titania was independent of the chemistry of the material surface, and dependent only on the optimal surface topography of nanophase ceramics. This conclusion was also drawn in another study by Webster and Ejiofor (2004), performed on low-passaged human osteoblasts in cultures on nanophase metals (Ti, Ti6Al4V and CoCrMo alloys).

However, this conclusion was not confirmed in our experiments performed on MG 63 cells in cultures on TiO_2 films (in anatase form) and on microscopic glass slides, where the cells spread and grew better on a TiO_2 surface than on glass slides of the same roughness (Vandrovcová *et al.* 2010). Similar results were obtained in rat calvarial osteoblasts in primary cultures on silicon substrates coated with TiO_2 in anatase, rutile and amorphous crystal phases (He *et al.* 2008). Although all three films were of similar roughness and topography, the cell spreading area, measured 8 hours after seeding, was largest on TiO_2 in anatase form. On anatase films, the cells also reached the highest cell numbers 36 and 72 hours after seeding. In addition, on day 7 and 14 after seeding, they displayed the greatest activity of alkaline phosphatase, i.e. an enzyme participating in bone tissue mineralization, and thus an important marker of osteogenic cell differentiation (He *et al.* 2008). These beneficial effects of anatase on cell colonization and cell phenotypic maturation were attributed to the highest surface hydrophilia of anatase (water drop contact angle about 60°) compared with rutile and amorphous TiO_2 (contact angle ~90°, He *et al.* 2008). This explanation was further supported by Sawase *et al.* (2008), who described further enlargement of cell spreading area *in vitro* and also enhanced bone apposition *in vivo*, when the hydrophilicity of anatase was increased by being irradiated with ultraviolet light prior to seeding the cell or implanting it into the rabbit tibia. Thus, the positive effect of the material surface nanostructure on cell performance can be further enhanced by surface hydrophilia, which (in synergy with the nanostructure) induces the adsorption of cell adhesion-mediating

ECM molecules in advantageous spatial conformations, i.e. with specific amino acid sequences, serving as ligands for cell adhesion receptors exposed to these receptors (Bačáková *et al.* 2004, Bačáková and Švorčík 2008).

4.4. Novel bioactive films on the material surface

The physical and chemical properties of the surface of a material can also be modified by deposition of other materials (atoms, molecules, particles) in the form of continuous or micropatterned layers. A wide range of organic and inorganic materials can be used for modifying the material surface, such as entire ECM molecules and ECM-derived adhesion oligopeptides (i.e., ligands for cell adhesion receptors), fibrin, ceramics represented mainly by hydroxyapatite or tricalcium phosphate (for a review, see Bačáková *et al*. 2007a, Bačáková and Švorčík 2008, Filová *et al*. 2009b, Vagaská *et al*. 2010). Recently, we carried out an intensive study of the influence of layers made of carbon nanoparticles, namely fullerenes, nanotubes, nanocrystalline diamond, on adhesion, growth, viability, metabolic activity and osteogenic cell differentiation, and we found that these layers are promising for surface modifications of bone implants (Bačáková *et al.* 2007b, Grausová *et al*., 2008a,b, 2009a,b, Vandrovcová *et al.* 2008; for a review see Bačáková *et al*. 2008).

4.4.1. Fullerenes

Fullerenes are spherical molecules made of carbon atoms, which are of a hollow cage-like shape similar to clathrin-coated vesicles in cells. This suggested the idea of using them for drug and gene delivery, or even for constructing artificial cellular organelles (for a review, see Bačáková *et al.* 2008). These carbon allotropes were discovered by Kroto *et al.* (1985), and have been considered as promising for various biomedical applications. However, it should be pointed out that fullerenes can be cytotoxic. For example, after irradiation with

visible or ultraviolet light, they convert oxygen molecules into highly reactive radicals, which can damage the cell membrane, various intracellular molecules, enzymes, and also DNA. This property of fullerenes can be utilized in photodynamic therapy against tumours, viruses and bacteria resistant to multiple drugs. On the other hand, fullerenes can also act as potent radical scavengers (for a review, see Bačáková *et al.* 2008). An interesting issue is the promoting action of fullerenes C_{60} on chondrogenesis, probably due to stimulatory effects of these compounds on proteoglycan synthesis (Tsuchiya *et al*. 1995). Some derivatives of fullerenes also have affinity to the bone tissue. For example, Gonzalez *et al.* (2002) found that some fullerene derivatives can affect bone tissue mineralization. These authors investigated the interaction between a bisphosphonate fullerene $C_{60}(OH)_{16}$AMBP, i.e. (4,4-bisphosphono-2-(polyhydroxyl-1,2-dihydro-1,2-methanfullerene(60)-61-carboxamido)butyric acid) and HAp, i.e., an important inorganic component of the bone tissue. $C_{60}(OH)_{16}$AMBP has strong binding sites for HAp, which was manifested by capturing HAp from a solution and by its lower availability for bone tissue mineralization. On the other hand, this new knowledge on bone-vectored compounds can be useful in bone radiation therapy, where the radionuclide-containing fullerene can be bound to the diseased site in the bone, enabling a low dose to be used to avoid damage to the non-diseased tissue.

Fullerenes can be deposited on the material surface as nanostructured layers of various thicknesses, which can be controlled by the temperature and time of deposition. These layers can also be deposited through a metallic mask in micropatterned forms, which are useful in applications where regionally-selective cell adhesion and directed growth is desirable (Fig. 2), e.g. in tissue engineering, microarrays for advanced proteomics and genomics, and constructing biosensors (Grausová *et al.* 2008a, 2009b; Vandrovcová *et al.* 2008). In addition, fullerene molecules can be combined with other materials, particularly atoms of Ti, Co or Ni,

and form binary fullerene-metal composites, also promising for biomedical applications (Vandrovcová *et al.* 2008, Vacík *et al*. 2010; for a review, see Bačáková *et al.* 2008).

4.4.2. Carbon nanotubes

Carbon nanotubes are cylindrical molecules made of carbon atoms. They exist in two basic forms: single-walled nanotubes (SWNT; formed by one cylindrical graphene sheet) and multi-walled nanotubes (MWNT; containing two or more concentrically arranged graphene sheets) (Iijima 1991, Dresselhaus *et al*. 1996). Carbon nanotubes can also be cytotoxic under some circumstances. In the same manner as fullerenes, carbon nanotubes can cleave molecular oxygen into free radicals, which can induce oxidative stress, inflammation, changes in the structure of proteins such as enzymes, extracellular matrix molecules and cell membranes. They can also disrupt DNA (Cui *et al*. 2005, Davoren *et al*. 2007, Kisin *et al.* 2007, Zhang *et al*. 2007). However, several studies have cast doubt on the cytotoxic action of nanotubes (Chen *et al.* 2006, Dumortier *et al.* 2006, Yehia *et al.* 2007, Zhu *et al*. 2006). These controversial results may be explained by the use of nanotubes of different size, purity, functionalization, water solubility and different tendency to form agglomerates (for a review, see Bačákova *et al*. 2008). Many studies have focused on applications of carbon nanotubes for tissue engineering in neural and also vascular systems, mainly as components of scaffolds for cell colonization and functioning (Matsumoto *et al*. 2007, Mattson *et al*. 2000, MacDonald *et al*. 2005). However, carbon nanotubes are also promising for bone tissue engineering. Abarrategi *et al.* (2008), for example, tested MWNT on composites with chitosan loaded by human bone morphogenetic protein-2, and these authors found osteoinductive effects of nanotubes on mouse myoblast cell line C2C12. A positive effect of nanotubes in combination with HAp on spreading and phenotypic maturation of human osteoblasts has also been described (Balani *et al.* 2007).

4.4.3. Nanocrystalline diamond

Nanocrystalline diamond has become widely investigated for its excellent properties, such as low friction coefficient, chemical stability and particularly high biocompatibility (Elam 2004, Tjong and Chen 2004, Amaral *et al*. 2008, Bačáková *et al*. 2007b, Grausová *et al*. 2008a,b; 2009a,b). Unlike the two groups of carbon allotropes mentioned above, nanocrystalline diamond is non-cytotoxic (Schrand *et al.* 2007, Aspenberg *et al*. 1996) and non-immunogenic (Tang *et al*. 1995, Nordsletten *et al*. 1996). These properties of nanocrystalline diamond enable its application not only in electronics and optics but also in biology and medicine. Due to their hardness, nanodiamond films proved to be suitable for coating the heads and cups of artificial joints (Papo *et al.* 2004). These films also supported adhesion, growth and differentiation of bone cells (Bajaj *et al*. 2007, Amaral *et al.* 2008). Thus, nanocrystalline diamond could be applied for coating the parts of joint prostheses or dental implants that anchor the bone with the surrounding bone tissue.

4.4.4. Diamond-like carbon

Another important carbon-based material is diamond-like carbon (DLC), also referred to as amorphous carbon. There are seven different forms of amorphous carbon materials, differing in the content of hydrogen (hydrogen-free or hydrogenated with various hydrogen concentrations), hybridization (sp^2 or sp^3) or presence of additional non-carbon atoms, such as metals (W, Ti) or non-metallic elements (Si, O, N, F, B) (Fraunhofer Institute, Name Index of Carbon Coatings).

DLC displays some of the special properties of diamond, such as high hardness, chemical inertness, and good tribological characteristics. Further, DLC elicited no inflammatory response *in vitro* and induced no histopathological changes *in vivo* (Schroeder *et al.* 2000, Bruinink *et al*. 2005). Similarly as nanocrystalline diamond films, DLC can be

deposited on orthopedic implants in order to prevent the release of metal ions (Dearnaley 1993, Dowling *et al.* 1997). Most DLC-based materials, particularly those containing only C and/or H atoms, act as bioinert, i.e. not supporting cell adhesion, and thus they have been utilized for coating blood contacting devices, such as heart valves or coronary stents, in order to prevent thrombus formation (Grill 2003). However, in some applications, it is necessary to promote cell adhesion (e.g. for better integration of bone implants with the surrounding bone tissue). In such cases, DLC and related materials (e.g. hydrocarbon plasma polymers) have been rendered bioactive by adding titanium atoms (Bačáková *et al*. 2001, Grinevich *et al*. 2009).

5. Conclusion and further remarks

A wide variety of materials have been used for constructing bone implants and replacements, such as synthetic and natural polymers, ceramics, metals and their composites. Bone replacements can be constructed in two forms: "two-dimensional", i.e. interacting with cells only at the implant surface, and "three-dimensional", allowing ingrowth of the bone tissue inside the implant, which is usually combined with degradability of the material and its gradual replacement by regenerated bone tissue. Nevertheless, for both forms of implants, the physicochemical properties of the material at the cell-material interface are decisive for the cell-material interaction. There are many ways to modify the surface of a material in order to enhance its attractiveness for cell colonization and osteogenic cell differentiation. Creating the nanostructure of the surface, especially in combination with surface hydrophilia, seems to be the most efficient of these approaches.

The research in the field of bone tissue replacement and regeneration is endless. What usually happens is that new materials and new modifications to them are found, which are at least slightly better than the systems of the previous generation. Despite all the efforts of

scientists, innovations are delayed by the occurrence of new complications, such as new diseases, allergies or problems related to long-term exposure of the organism to artificial tissue replacements. Everybody has to accept that death is inseparable from our destiny, and that it will occur in exactly 100% of cases. Thus, any effort to avert death in all circumstances seems to be a completely unscientific activity, because death comes to all of us mortals (Komárek and Djakow 2010). However, biomaterial science and tissue engineering are capable of improving the quality of the life that remains between the onset of a disease and death. This is a desirable contribution, and is of great importance for an advanced human society.

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Figures

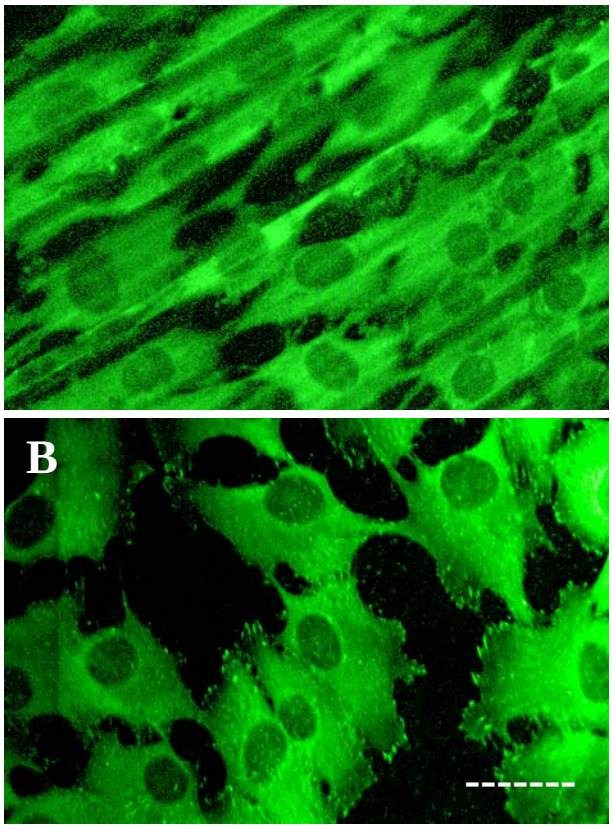

Fig. 1. Immunofuorescence of vinculin, an integrin-associated focal adhesion protein, in human osteoblast-like MG 63 cells in 4-day-old cultures on carbon fibre-reinforced carbon composites (CFRC) with an unmodified surface (**A**), and with the surface ground using metallographic paper, coated with pyrolytic graphite, again ground using metallographic paper and polished with diamond 3/2 (**B**) **A**: Less spread cells on pristine composites of microscale surface roughness (R_a = 6.5 µm, S = 42 µm), elongated along the carbon fibres bulging on the material surface, and without visible vinculin-containing focal adhesion plaques. **B**: Well-spread polygonal cells with clearly developed vinculin-containing focal adhesion plaques in cultures on modified composites with submicron-scale surface roughness (R_a = 0.67 µm, S = 81 µm).Bio-Rad MRC600 confocal laser scanning microscope, oil immersion 60× objective, (numerical aperture = 1.4), excitation wavelength 488 nm. Scale bar = 50 µm. R_a: departures of the roughness profile from the mean line (i.e., size of the irregularities), S: mean spacing of the adjacent local peaks (i.e., distances among the irregularities).

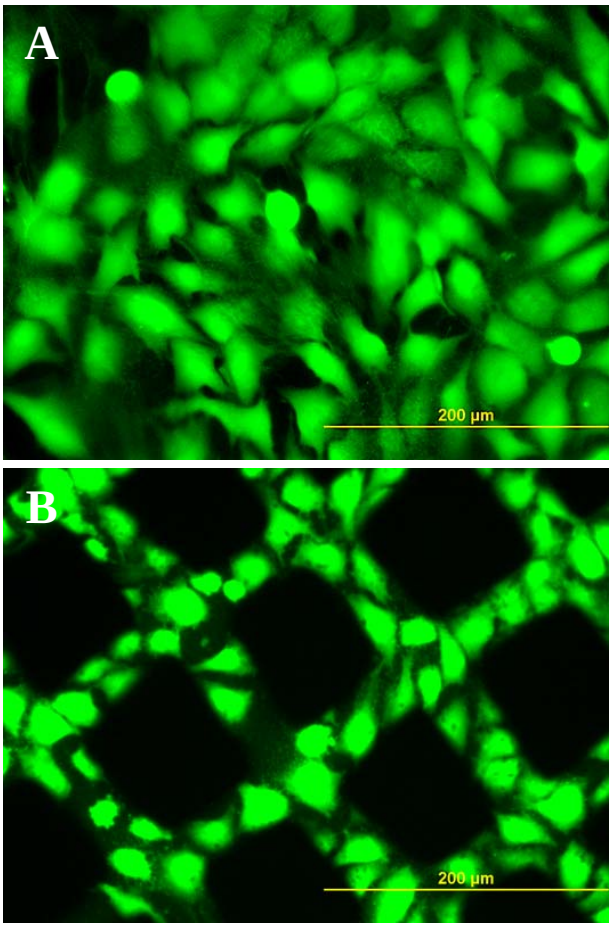

Fig. 2. Human osteoblast-like MG 63 cells in 7-day-old cultures on fullerene C_{60} layers micropatterned with prominences 128 ± 8 nm in height (**A**) or 1043 ± 57 nm in height (**B**). **A**: homogeneous distribution of cells on the surfaces with relatively low prominences; **B**: preferential localization of cells in grooves among relatively high prominences. Cells stained with LIVE/DEAD viability/cytotoxicity kit (Invitrogen). Olympus IX 51 microscope, DP 70 digital camera, obj. 20x, bar = 200 µm