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# CARBON NANOPARTICLES AS PROMISING COMPONENTS OF MATERIALS FOR BONE TISSUE ENGINEERING

**PhD Thesis**

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## Contents

# 1. INTRODUCTION

## 1.1. What is tissue engineering?

The destruction of an organ or tissue occurs in millions of patients every year. The biomedical field dealing with tissue regeneration is known as tissue engineering. Important roles in tissue engineering are played by biomaterials and cells. A biomaterial is a material that is used and adapted for a medical application.

Biomaterials can be divided into four major classes of materials: polymers, metals, ceramics and carbon materials. Two different materials combined together create a composite. The first step in tissue engineering is *in vitro* formation of a tissue construct by placing the cells and the biomaterial in a metabolically and mechanically supportive environment with growth media in a bioreactor, in which the cells proliferate and produce an extracellular matrix. In the second step, the construct is implanted in the place in the body where the tissue needs to be regenerated (Ratner *et al*., 2004). The key processes in tissue engineering are (1) cell proliferation, sorting and differentiation, (2) extracellular matrix production and organisation, (3) degradation of the scaffold and growth of tissue (Bianco and Robey, 2001; Blau *et al*., 2001).

Three generations of biomaterials can be differentiated. The "first generation" of modern biomaterials consisted of widely available industrial materials that had not been developed specifically for medical use. They were selected due to a desirable combination of physical properties suitable for a specific clinical use, and they were intended to be bioinert. Pyrolytic carbon, developed in the 1960s as a coating material for nuclear fuel particles and now widely used in mechanical heart valve substitutes, exemplifies one of the first biomaterials whose formulation was studied, modified and controlled according to engineering and biological principles specifically for medical applications (Bokros *et al*., 1977). The "second generation" of biomaterials was intended to elicit a nontrivial and controlled reaction with the tissues into which they were implanted, in order to induce a desired therapeutic advantage. In the 1980s, biomaterials were in clinical use in orthopedic and dental surgery as various compositions of bioactive glasses and ceramics (Hench and Polak, 2002). The second generation of biomaterials included the development of resorbable biomaterials with variable rates of degradation. Biomaterials of the "third generation" are intended to stimulate highly precise reactions with proteins and cells at the molecular level.

Nanotechnology has opened new possibilities for improving tissue regeneration. (Ebbesen and Jensen, 2006; Price *et al*., 2004; Webster *et al*., 200a,b; Webster and Smith 2005)

# 2. MATERIALS USED IN MEDICINE

## 2.1. Polymers

Polymers represent the largest class of biomaterials. They can be derived from natural sources, or from synthetic organic processes. Polymers can be used as an implant in a pure form or are often used in the form of composites, for example with carbon nanotubes (Bačáková *et al*., 2007, 2008; MacDonald *et al*., 2005; Shi *et al*., 2007).

### 2.1.1. Natural polymers

Natural polymers include plant materials such as cellulose, sodium alginate or natural rubber, animal-derived materials such as tissue-based heart valves and sutures, collagen, glycosaminglycans, heparin, hyaluronic acid and other natural materials (Ratner *et al*., 2004). An important natural material that is promising for various biomedical applications is chitosan, a polysaccharide with amine groups, derived from fungi, arthropods, moluscs and other organisms (Abarategi *et al.* 2008; Bhattarai *et al*., 2005; Smith and Ma, 2004). However, a major drawback of natural materials is their potential immunogenicity and the risk of disease transmission.

### 2.1.2. Synthetic non-degradable polymers

#### 2.1.2.1. Silicones

Silicones are synthetic polymers whose backbone is made of repeating silicon to oxygen bonds. Silicon atoms can be bonded to organic groups, usually to methyl groups. Many other groups, e.g., phenyl, vinyl and trifluoropropyl, can be substituted for the methyl groups. The bonding of organic groups to an inorganic backbone regulates the chemical properties of silicones, making them usable as fluids, emulsions, compounds, resins, and elastomers in numerous applications. Silicones are highly biocompatible and can therefore be successfully used in many pharmaceutical and medical applications (Ratner *et al*., 2004).

#### 2.1.2.2. Hydrogels

Hydrogels are water-swollen, cross-linked polymeric structures containing covalent bonds produced by the simple reaction of one or more comonomers (Peppas, 1987). The most used biomedical hydrogel is water-swollen, cross-linked poly(hydroxyethyl methacrylate) (PHEMA), which was introduced as a biological material by Wichterle and Lim (Wichterle and Lim, 1960). This hydrogel is inert to normal biological processes, resistant to degradation, permeable to metabolites, not absorbed by the body, biocompatible, sterilizable with heat without damage, and it can be prepared in many types of shapes and forms. One of the earliest biomedical applications of hydrogels was in the construction of contact lenses (Peppas and Yang, 1981).

Hydrogels offer good mechanical stability, a favorable refractive index, and high oxygen permeability. Other potential applications of hydrogels include artificial tendon materials, wound-healing bioadhesives, artificial kidney membranes, articular cartilage, artificial skin, maxillofacial and sexual organ reconstruction materials (Ratner *et al*., 2004). Recently, hydrogels have been studied in regeneration of the central nerve system (Nisbet *et al*., 2007), vocal fold regeneration (Sahiner *et al*., 2008) and bladder tissue regeneration (Adelöv and Frey, 2007).

#### 2.1.2.3. Plastics

Plastics such as polytetrafluorethylene (PTFE), polyvinyldifluoride (PVDF), polyethylene terephthalate (PET), polyurethane (PU and polypropylene (PP) have been widely studied and used in vascular tissue engineering (Cikirikcioglu *et al*., 2008; Desgranges *et al*., 2001; Mary *et al*., 1998; Seifalian *et al*., 2003; Zhang *et al*., 2006),

Recently it has been found that these materials, usually in their modified form, can also be successfully used for regenerating bone (Bačáková *et al*., 2007; Chang *et al*., 2008; de Macedo *et al*., 2008; Jung *et al*., 2008; Laureano *et al*., 2007) or cartilage (Buxton *et al*., 2007; Mola *et al*., 2007; Moroni *et al*., 2007; Omori *et al*., 2004; Wang *et al*., 2008).

### 2.1.3. Synthetic degradable polymers

Degradable implants do not have to be removed surgically once they are no longer needed. In advanced tissue engineering, degradable materials can serve as a temporary support for cells taking part in the regeneration of damaged tissue. The degradation products of the implant are released into the patient's body, so it is very important to test the potential toxicity of their degradation products. Degradation refers to a chemical process of the

cleavage of covalent bonds. Hydrolysis is the most common chemical process by which polymers degrade, but degradation can also occur via oxidative and enzymatic mechanisms (Ratner *et al*., 2004).

#### 2.1.3.1. Poly(ether esters)

Polydioxanone (PDS) is a poly(ether ester) made by ring-opening polymerization of *p*-dioxanone monomer. Monomers of PDS present low-toxicity *in vivo* and this polymer has therefore attracted increasing interest in the medical and pharmaceutical field. PDS has been used in orthopaedics for bone pins (Heller *et al*., 2002). It has also been studied in nerve regeneration (Shen *et al*., 2001) and cartilage regeneration (Bönisch and Mink, 2000).

#### 2.1.3.2. Poly(hydroxybutyrate) (PHB), polyhydroxyvalerate (PHV)

PHB and PHV are a group of bioresorbable polyesters that are derived from microorganisms *Alcaligenes eutrophus* or *Bacillus megaterium*. PHB and PHV are products of carbon assimilation from glucose or starch (Steinbüchel, 2002). These polymers are relatively stable under physiological conditions (pH 7.35, 37°C). Their rate of degradation can be modified by the copolymer composition, but all members of this family of polymers require several years for complete resorption *in vivo*. *In vivo*, PHB degrades to D-3-hydroxybutyric acid, which is a normal component of human blood (Miller and Williams, 1987). PHB and PHV polymers have been considered in several biomedical applications, such as controlled drug release and tissue regeneration (Cheng *et al*., 2006; Galgut *et al*., 1991; Wang *et al*., 2001).

#### 2.1.3.3. Poly(glycolic acid) (PGA) and poly(lactic acid) (PLA)

These materials are the most commonly used synthetic degradable polymers. PLA and PGA are considered as safe, nontoxic and biocompatible materials. Currently available and approved products include sutures, membranes for dentistry, bone pins, and implantable drug delivery systems (Ratner *et al*., 2004). From many studies, including those performed in our laboratory, it is known that both PLA and PGA give good support for the growth of bone cells (Hasegawa *et al*., 2007; Katanec *et al*., 2004; Liu *et al*., 2006; Pamula *et al*., 2008; Smith *et al*., 2007; Sun *et al*., 2007).

However, these polymers have some significant disadvantages: (1) In tissue culture experiments, the cells often did not attach to PLA or PGA surfaces and did not grow as well as on the surface of other materials, e.g. tissue culture polystyrene, indicating that these

polymers are not perfect substrates for cell growth *in vitro* (Pamula *et al*., 2008). (2) In addition, the degradation products of PLA and PGA are relatively strong acids. When these degradation products accumulate at the implant site, a delayed inflammatory response is often observed months to years after implantation (Athanasiou *et al*., 1998; Bergsma *et al*., 1995; Törmälä *et al*., 1998). (3) Finally, the mechanical strength of these materials is usually insufficient to match the requirements of bone tissue engineering, especially concerning the construction of load-bearing bone implants (Pamula *et al*., 2008).

#### 2.1.3.4. Polycaprolactone (PCL)

PCL is a semicrystalline polymer. It degrades more slowly than poly(lactic acid) (PLA), and can therefore be used in drug delivery devices that remain active for over one year. ε-caprolactone and polycaprolactone are currently regarded as nontoxic and tissue-compatible materials (Ratner *et al*., 2004). PCL has been shown to be useful in the regeneration of osteochondral tissue (Hsu *et al*., 2007; Schumann *et al*., 2007; Wan *et al*., 2008) and the urinal bladder (Yu *et al*., 2007). On the other hand, Jeans *et al*. demonstrated that this material is not suitable for repairing nerves (Jeans *et al*., 2007).

#### 2.1.3.5. Polyanhydrides

Polyanhydrides are reactive and hydrolytically unstable polymers. Aliphatic anhydrides degrade within days, whereas some aromatic polyanhydrides degrade over several years. Their high chemical reactivity is both an advantage and a limitation of polyanhydrides (Leong *et al*., 1986; Tamada and Langer, 1993). Polyanhydrides have been shown to be useful as drug deliverers in the treatment of chronic osteomyelitis and in the prophylaxis of bone infection (Chen *et al*., 2007), and also in periodontal regeneration (Reynolds *et al*., 2007).

#### 2.1.3.6. Poly(amino acids)

Polypeptides and proteins are considered as promising candidates for attachment of drugs, cross-linking agents, or pendent groups that can be used to modify the physicomechanical properties of the polymer (Ratner *et al*., 2004). In addition to the natural peptides and proteins, these molecules, e.g., human elastin-like polypeptides, can be prepared as recombinant, i.e. synthetically, using microorganisms (Bellingham *et al*., 2003). In tissue engineering, they can be used as an anchor for cell membrane receptors, and can thus regulate cell adhesion to the biomaterial (Bačáková *et al*., 2004, 2007; Gobin and West 2003).

### 2.1.3.7. Polycyanoacrylates

Polycyanoacrylates are used as bioadhesives, usually as a dental adhesive. These materials undergo spontaneous polymerization under room temperature in the presence of water, and their toxicity and erosion rate differ with the length of their alkyl chains (Gombotz and Pettit, 1995). The monomers are very reactive compounds and cause high toxicity. During degradation of polycyanoacrylates, the release of formaldehyde evokes significant inflammation in the surrounding tissue (Ratner *et al*., 2004).

### 2.1.3.8. Polyphosphazenes

Polyphosphazenes are relatively rarely used polymers, whose backbone consists of nitrogen-phosphorus bonds. These polymers are at the interface between inorganic and organic polymers and have unusual material properties, e.g. high thermal stability. This material is known to be biocompatible and promising for use in bone tissue regeneration (Laurencin *et al*., 1993).

## 2.2. Metals

Metals are strong, and have good mechanical properties which are needed for hard tissue surgery. Commonly-used metals are stainless steel, Co-Cr alloys and Ti alloys, such as Ti-Al-V. Several types of stainless steels are available for implant use; in medical practice, the most common is 316L steel. This type of steel has less than 0.030 wt% carbon in order to reduce the possibility of *in vivo* corrosion. The 316 alloy consists predominantly of iron (60-65%) with chromium (17-20%) and nickel (12-14%), and minor amounts of nitrogen, manganese, molybdenum, phosphorus, silicon and sulphur. The main function of chromium is to permit the development of corrosion-resistant steel by forming a strongly adherent surface oxide ($Cr_2O_3$). Molybdenum and silicon have the role of ferrite stabilizers. The main reason for the low carbon content in 346L is to improve corrosion resistance. If the carbon content of the steel significantly exceeds 0.03%, there is increased danger of carbide formation (Brunski, 1983). The good biocompatibility of titanium is explained by a layer of $TiO_2$ on the surface, which promotes cell adhesion (Lausmaa *et al*., 1985).

Titanium prostheses are widely used in dental and bone surgery. There are many studies which show excellent biocompatibility of titanium with bone cells. For example, Takemoto and coworkers demonstrated that this metal can be used for spinal interbody fusion (Takemoto *et al*., 2007). Smith *et al*. have prepared a composite of nanostructured titanium and PGA which showed positive effects on osteoblast cell growth (Smith *et al*., 2007).

Though titanium alloys are biocompatible and nonimmunogenic, they do not exhibit antibacterial activity, and these materials therefore exhibit an increasing bacterial colonization tendency (Yoshinary *et al*., 2000). In addition, metallic implants, including titanium, are relatively rigid and heavy in comparison with bone tissue, and this can lead to damage and aseptic loosening of the adjacent bone tissue, if these materials are used as bone implants (Bačáková *et al*., 2001; Blazewicz *et al*., 1997; Merolli *et al*., 1999).

### 2.3. Carbon materials

Carbon is a very important and successful component in tissue engineering, and it occurs in many forms. Carbon has been considered as biocompatible, and it is often functionalized with different chemical groups, which can enhance the biocompatibility of carbon materials. The use of carbon materials as artificial grafts started in 1968, with the replacement of a heart valve with a mechanical device made of pyrolitic carbon. Pyrolytic carbon is man-made, and does not occur in nature. Pyrolytic carbon is one of the most successful biomaterials in application as well as in function. Among the materials available for mechanical heart valve prostheses, this carbon allotrope has the best combination of blood compatibility, physical and mechanical properties, and durability. However, chronic anticoagulant therapy is needed for patients with mechanical heart valves.

Chinn and coworkers reexamined the adsorption of albumin and fibrinogen on pyrolytic carbon surfaces and noted that relatively large amounts of fibrinogen were adsorbed. They speculated that the adsorbed fibrinogen was rapidly converted to a non-elutable form (Chinn *et al*., 1994). Pyrolytic carbon also proved to be a suitable coating for composites with a carbon matrix reinforced with carbon fibres, so-called carbon fiber-reinforced carbon composites (CFRC). Originally, these materials were developed for industrial applications, but due to their advantageous mechanical and other physical properties, they have also been considered promising for bone tissue engineering (for a review, see Bačáková *et al*., 2001). Pyrolytic carbon strengthens the surface of CFRC, prevents the release of particles from these materials and enhances their colonization with human osteoblast-like MG 63 cells (Starý *et al*., 2003a,b).

Nowadays, the scientific fields of nanotechnology and tissue engineering are concentrating on carbon materials of nanosize structures. There are many studies about the biological effects of carbon nanofibers, carbon nanotubes and related structures, such as carbon nanohorns, fullerenes and nanodiamonds in the form of a powder or films (for more details, see chapter 4).

## 2.4. Biocompatible ceramics and glasses

Biocompatible ceramics and glasses are used in hard tissue surgery and dentistry. It has been repeatedly shown that bone cells have a high bonding affinity to these materials; there are also some specially modified bioglasses that are suitable for soft tissues (Ratner *et al*., 2004). The main characteristic of bioactive glass and ceramics is a time-dependent, kinetic modification of the surface that occurs upon implantation. The surface forms a hydroxyapatite which provides the bonding interface with the tissues. The affinity of bone cells to bioactive glass containing $SiO_2$, $Na_2O$, CaO, and $P_2O_5$ was first shown by Hench in 1972 (Hench *et al*., 1972). Hydroxyapatite (HAP) in ceramics has been used for more than 20 years. It is applied mainly in dentistry and bone surgery.

Staffa *et al*. used hydroxyapatite scaffolds in neurosurgery in patients with cranial fractures. The artificial cranium was made from epoxy resin by stereolithography. The prosthesis was built on this model using a ceramic sintering process, and was an exact copy of the missing part of the cranium with its typical curvature, dimensions, margins, irregularities and thickness. The prosthesis was made of hydroxyapatite with high interconnective porosity (40-70%), and has around 60-70% of macropores (diameter greater than 150 μm) and around 20% micropores (diameter less than 10 μm). The "hydroxyapatite cranium" has been applied in many patients, and has shown perfect biocompatibility, with no cases of infection, rejection or spontaneous prosthesis fragmentation (Staffa *et al*., 2007).

Rouahi *et al*. studied the *in vitro* influence of microporous hydroxyapatite ceramic on serum protein adsorption and SaOs-2 human bone cell attachment. These authors discovered that SaOs-2 cells proliferated at a relatively high rate, and adhered strongly to the microporous hydroxyapatite. In addition, more fibronectin and serum albumin were adsorbed to the microporous hydroxyapatite than to the control plastic coverslips (Rouahi *et al*., 2006).

Another important member of the calcium phosphate group, used in bone tissue engineering, is tricalcium phosphate (TCP). TCP ceramics have often been preferred over HAp because of their high dissolution rate, which has been reported to facilitate new bone tissue formation under *in vivo* conditions, e.g., in experimental mandibular defects in sheep or minipigs (Gatti *et al*., 1990; Jensen *et al*., 2007). On the other hand, the higher solubility of TCP can be associated with a higher release and local concentration of calcium and phosphate ions, which can act toxically on the surrounding cells (Detsch *et al*., 2008; John *et al*., 2003; Yamada *et al*., 1997).

In our earlier study, performed in a conventional static cell culture system, adhesion substrates for human osteoblast-like MG 63 cells, made of beta-TCP, induced strong alkalization of the cell culture media, followed by death of the cells (Bačáková *et al*., 2004).

# 3. CELL-MATERIAL INTERACTION

## 3.1. Extracellular matrix as a natural cell carrier

Tissues are built of cells and extracellular matrix (ECM). Extracellular matrix is composed of a variety of proteins and polysaccharides that are secreted locally by cells, e.g., by fibroblasts, chondroblasts, osteoblasts, or vascular smooth muscle and endothelial cells, and are assembled into an organized meshwork. The diversity of composition, organization and distribution of ECM depends not only on differential gene expression for the various molecules in specific tissues, but also on the existence of differential splicing and posttranslational modifications (Gaudry *et al*., 2008; Saad *et al*., 2008; White, 2008).

The matrix is made up of two main classes of extracellular molecules: (1) Glycosaminoglycans are polysaccharides covalently bound to protein to form proteoglycans. (2) Fibrous proteins, such as collagen, elastin, fibronectin and laminin, have structural and adhesive functions. The extracellular matrix is specific for each tissue. In bones and teeth, the ECMs also contain an inorganic component, e.g. calcium compounds, in order to produce their mechanical strength.

ECM influences many events in cell behavior, such as cell adhesion/de-adhesion, cell phenotype, cell shape, polarity, cytoskeletal reorganization, cell migration, proliferation, differentiation, functioning and programmed cell death. The cell-ECM interaction occurs through receptors for specific ECM molecules on the cell membrane, e.g. integrins. In most tissues, the ECM is constantly turning over and being remodeled. Enzymes called metalloproteinases are responsible for the degradation of ECM proteins (Cornelius *et al*., 1998).

## 3.2. Extracellular matrix as the main mediator of cell-material interaction

Most tissue-derived cells used for colonization of biomaterials and for constructing bioartificial tissues and organs are anchorage-dependent. They require attachment to a solid surface for their viability, growth, migration and differentiation. Cell behaviour therefore

depends strongly on surface characteristics. Two mechanisms of cell adhesion to artificial materials are known:

(1) Non-receptor mediated cell adhesion refers to non-specific cell-material interactions through weak chemical bonding, such as hydrogen bonding, electrostatic or ionic interactions between molecules on the cell membrane and biomaterial without the presence of ECM proteins. However, this interaction does not deliver specific signals into the cells, and does not support stable and long-term cell adhesion, growth, viability and other cell function (Bačáková *et al*., 2000a,b.)

(2) Functional receptor-mediated and signal-transmitting cell adhesion to a biomaterial is mediated by ECM proteins adsorbed on the material surface (Garcia *et al*., 1999; Groth *et al*., 1999). These proteins, such as vitronectin, fibronectin, collagen or laminin, adsorb spontaneously to the material from the cell culture media *in vitro* or from the body fluids *in vivo*. The cells bind specific amino acid sequences of ECM proteins, such as Arg-Gly-Asp (RGD), which is recognized by many types of cells (Bačáková *et al*., 2004), or other cell-specific sequences, such as Arg-Leu-Asp-Val (REDV) preferred by vascular endothelial cells (Massia and Hubbell, 1992), Val-Ala-Pro-Lys (VAPG) preferred by vascular smooth muscle cells (Gobin and West, 2003), Lys-Gly-Ala-Gly-Asp-Val (KGAGDV) preferred by polymorphonuclear leukocytes (Gresham *et al*., 1992) or Lys-Arg-Ser-Arg (KRSR) preferred by osteoblasts (Nelson *et al*., 2006). These sequences are recognized by adhesion receptors on the cell membrane. The most known, best described and systemized are adhesion receptors of the integrin superfamily. Integrins are heterodimeric transmembrane glycoproteins consisting of one alpha chain and one beta chain. The function of integrins is dependent on calcium, which binds to the alpha subunit (for a review, see Bačáková *et al*., 2004). After a cell binds to a biomaterial, the integrin receptors are recruited into distinct dot-like nano- or microdomains called "focal adhesion plaques", "focal adhesion sites" or "focal adhesions". Focal adhesion proteins, such as talin, alpha-actinin, vinculin, filamin or paxillin act as linkers between the integrin receptor and the cytoplasmic actin cytoskeleton, which is associated with nuclear membrane, membranes of organelles and various enzymes (Aplin, 2003; Moiseeva, 2001; Hemler, 1998; Horton, 1997; Hynes, 1999). It was found that there are also other cell-matrix adhesion receptors than integrins, often based on saccharide molecules (Aplin, 2003; Dee *et al*., 1998; Moiseeva 2001; Park *et al*., 1998). For example, a heparan sulphate on osteoblasts recognizes a bone specific oligopeptide Lys-Arg-Ser-Arg (Dee *et al*., 1998), or an asialoglycoprotein-based receptor on hepatocytes binds a galactose ligand (Park *et al*., 1998).

The adsorption of cell-adhesion mediating ECM proteins and the subsequent cell-material interaction is strongly dependent on the physicochemical properties of the material surface, such as the presence of various chemical functional groups, polarity, wettability, electrical charge and conductivity, surface roughness and topography. The mechanical properties of the adhesion substrate, i.e. its rigidity or elasticity, are also crucial for the cell-material interaction (Ingber *et al*., 1995; Huang *et al*., 1998; Wang *et al*., 2001).

As regards ***chemical functional groups***, the surface of a biomaterial can be modified with oxygen-containing groups (hydroxyl, carboxyl, carbonyl, ester, aldehyde, etc.) or amine groups, which have been repeatedly reported to improve cell adhesion and growth (for a review, see Bačáková *et al*., 2000a,b; 2001, 2004). The mechanism is increased wettability of the material surface, and in the case of $NH_2$ groups, also a positive electrical charge of this surface.

As regards ***polarity and wettability,*** cell adhesion is supported by increasing the polar component of the free energy of the material surface, which leads to an increase in surface wettability. On highly hydrophobic surfaces (e.g., water drop contact angle more than 90°), the cell adhesion-mediating proteins are adsorbed in a rigid form resistant to reorganization by the cells, and their aspecific amino acid sequences are not accessible for cell adhesion receptors. In addition, highly hydrophobic surfaces preferentially adsorb albumin, which is non-adhesive for cells (Bačáková *et al*., 2004; Altankov *et al*., 1996; Groth *et al*., 1999). However, it is generally known that there is maximal cell adhesion to moderately hydrophilic surfaces. Highly hydrophilic surfaces (contact angle e.g. from 0° to 30°), do not allow stable adsorption of proteins, and thus disable subsequent cell attachment, or cause detachment of cells at an early stage of culture (Bačáková *et al*., 2007; Clem *et al*., 2008; Vandrovcová *et al*., 2008).

A positive ***electrical charge*** and ***electrical conductivity*** are other desirable properties of the adhesion substrate. They improve the adhesion, growth and differentiation of various cell types, such as osteoblasts or neurons. Like moderate wettability, the positive charge induces adsorption of cell adhesion-mediating molecules from biological fluids in an amount and geometrical conformation that makes them accessible to the cell adhesion receptors. Electrical conductivity of the cell adhesion substrate enables effective stimulation of the cell with an electrical current, and this leads to an increase in intracellular calcium, followed by the expression of appropriate genes and desirable cell functioning, e.g., synthesis and deposition of mineralized bone matrix by osteoblasts, or signal transmission by neurons (Bačáková *et al*., 2008; Supronowicz *et al*., 2002; Zanello *et al*., 2006). In contrast, a negative

surface charge repels cells, because it attenuates the adsorption of negatively charged cell adhesion-mediating ECM proteins (Lesný *et al*., 2006).

The ***surface roughness*** and ***topography***, i.e., the size, shape and spacing of the irregularities can markedly influence the attachment, spreading, proliferation and maturation of various types of cells on the material surface. Surface roughness in dimensions of micrometers or tens of micrometers has a dual effect on colonization of the material by cells. Earlier studies performed in our laboratory on carbon fibre-reinforced carbon composites (CFRC) showed that the adhesion and growth of human osteoblast-like MG 63 cells and rat aortic smooth muscle cells correlated negatively with increasing surface microroughness (Bačáková *et al*., 2001a). However, in our studies performed on MG 63 and endothelial cells cultured on nanocrystalline diamond films, and also in studies by other authors on various other materials, submicron and micron surface roughness promoted cell growth and differentiation (for a review, see Grausová *et al.*, 2008a,b).

Recently, special attention has been paid to the role of surface nanoroughness and nanotopography in cell-material interaction, i.e., the presence of irregularities smaller than 100 nm. The reason is that nanostructured materials mimic the architecture of the natural extracellular matrix and also the cell membrane (for more details, see chapter 4).

An interesting, important and as yet little investigated issue in cell-material interaction is the ***rigidity and elasticity*** of the material surface. Different types of tissue require different hardness of the biomaterial. For example, soft substrates are suitable for neuronal cells, while hard materials support adhesion of osteoblast cells (Engler *et al*., 2007). If the material is too elastic, flexible and irreversibly deformable, cells are not able to anchor on the substrate and thus undergo apoptosis. The underlying mechanism is that extremely soft materials are not able to withstand the tractional forces provided by the cell cytoskeleton (for a review, see Bačáková *et al*., 2004).

Similarly as in the case of natural ECM, artificial materials (through adsorbed ECM molecules) also influence the gene expression into the adhering cells. In anchorage-dependent cells, the first prerequisite for the survival, growth, differentiation and functioning of cells is their attachment and spreading in the material surface. The critical role of cell spreading in the further fate of a cell was intensively studied by Chen et al. They used endothelial cells cultured on a microfabricated biomaterial containing fibronectin-coated islands of various defined shapes and sizes on a micrometer scale. The size of the cells depended strictly on the size of the fibronectin islands. Cells on circular islands were circular, while cells on square islands were square in shape. When the cells were grown on substrates with fibronectin

islands of 10-30 μm, their proliferation was arrested, whereas larger islands (around 80 μm) strongly enhanced the cell proliferation (Chen *et al*., 1997). The main mechanism is the increasing tension of cytoskeletal fibres during cell spreading, which leads to expansion of the nuclear volume, enlargement of the nuclear pores, an increase in nuclear export, and also to the import of various extranuclear cell cycle-regulating factors, chromatin decondensation and thus increased accessibility of DNA to the replication machinery (Roca-Cusachs *et al.*, 2008). It was found that extremely small adhesion areas with a round cell lead to cell death, intermediate cell spreading provides maximal cell migration and proliferation, and very large cell adhesion areas can lead to switching between a proliferation and differentiation program (for a review, see Bačáková *et al*., 2004; Engler *et al*., 2004).

More advanced biomaterials are endowed on their surface with synthetic and chemically well-defined oligopeptidic or carbohydrate ligands for integrin or proteoglycan based receptors. These biomaterials are known as "bioactive" , "biomimetic", "biospecific" or "bioanalogous" templates of ECM. The great advantage of these biomaterials is cell specific binding and the avoidance of potential inflammatory reactions, thrombosis or device-associated infections, often associated with the use of entire ECM molecules, especially those of allogenous origin (Tang *et al*., 1998; Vande Vondele *et al*., 2003). Another option for controlling cell adhesion and behaviour is to incorporate and release in a controlled manner functional parts of natural growth factors, hormones, enzymes or synthetic cell cycle regulators (Brooks *et al*., 1997; Lutolf *et al*., 2003; Vella *et al*., 1999).

# 4. NANOTECHNOLOGY IN TISSUE ENGINEERING

## 4.1. Introduction to nanotechnology

Nanotechnology refers to the design, characterisation, production and application of structures, devices and systems that have novel physical, chemical and biological properties by controlling shape and size at the nanometer scale. Nanomedicine is the medical application of nanotechnology (Ebbesen and Jensen, 2006). Nowadays, nanomedicine exploits fields such as nanoscale surgery, tissue engineering, and targeted drug delivery (Emerich and Thanos, 2003; Haberzettl, 2002). Conventional materials with dimensions greater than 1 micron often do not invoke the proper cellular responses to regenerate tissue. Nanostructured biomaterials may be successful in tissue engineering, because they mimic the dimensions of the components of natural tissues. Nanostructured materials are defined as materials with

dimensions less than 100 nm in at least one dimension. Nanostructured surfaces are fabricated by using various methods such as electrical lithography (e.g., photolithography and electron beam lithography) and natural lithography (e.g., colloidal and block-copolymer lithography) (Curtis and Wilkinson, 1999; Nair *et al*., 2004; Park *et al*., 2003).

Biomaterials with nanostructured surfaces are widely studied in bone tissue engineering. Scaffolds for bone tissue engineering should be mechanically strong, biocompatible and osteoconductive. Stress and strain balances between an implanted material and the tissue are required for good bone regeneration. Polymeric materials or ceramics, widely used for fabricating these scaffolds, have several limitations due to insufficient mechanical properties. Polymeric materials are in general too elastic, while ceramics suffer from brittleness and difficulty of processing. Various nanoparticles could be very useful for improving biomaterials from several points of view. These particles can not only improve the mechanical properties of materials used for bone reconstruction. They also imitate the size and shape of organic and inorganic components of the bone, which are in nanodimensions. For example, nanoparticles of hydroxyapatite were incorporated into a poly(ethylene glycol) matrix. In this way, the brittleness of the ceramic material was avoided, the polymer was reinforced and the bioceramic nanoparticles mimicked the inorganic crystals present in the natural bone ECM (Liu *et al*., 1998). Another important feature of composite materials containing nanoparticles is that these particles provide the nanoroughness of the material surface that is essential for enhancing the adhesion, growth, maturation and function of osteoblasts (Sato and Webster, 2004). Metal nanoparticles have also been investigated for use in bone regeneration, because they markedly enhance the mechanical strength of the composite, and the material nanostructure provided by these particles also enhances cell adhesion. The *in vitro* and *in vivo* effects of nanophase aluminia and titania showed a significant increase in osteoblast adhesion (Price *et al*., 2003; Puckett *et al*., 2008; Sato *et al*., 2008).

In general, nanostructured surfaces are believed to improve the geometrical conformation of the adsorbed ECM proteins. Thus specific sites on these molecules are more accessible to the adhesion receptors on the cells (Kay *et al*., 2002; Webster *et al*., 1999; Webster and Smith, 2005). It has also been shown that composites containing PLGA and metal nanoparticles stimulate increased long-term osteoblast functions, including collagen synthesis, alkaline phosphatase (ALP) activity and calcium deposition (Webster and Smith, 2005). Webster *et al*. demonstrated that osteoblast adhesion is more dependent on the optimal

surface topography of the nanostructured composite than on material type (Webster *et al*., 1999).

Nanofibrous scaffolds also seem to be promising in tissue engineering. They are very suitable for bone tissue engineering not only due to their mechanical strength, which is essential for hard tissue, but also due to the fact that these nanofibrils imitate the organic component of the bone ECM, such as collagen and proteoglycans, which are essential for cell attachment, proliferation and differentiation. Nanofibrous scaffolds can be made from various materials, such as PLA, hydroxyapatite (Jeong *et al*., 2007), chitosan (Bhattarai *et al*., 2005), PCL (Li *et al*., 2005), PGA, collagen (Tian *et al*., 2008) or carbon (Price *et al*., 2004). PLA nanofibres improved adsorption of fibronectin and vitronectin from the media, promoting osteoblast attachment. The osteoblast attachment on nanofibrous PLA increased more than 1.7 times in comparison with unmodified PLA. PCL nanofibres demonstrated the ability to support and maintain multilineage differentiation of bone marrow-derived hMSCc *in vitro* into adipogenic, chondrogenic and osteogenic lineages (Li *et al*., 2005). Nanofibrous material containing chitosan and poly(ethylene oxide) blend promoted the attachment of human osteoblasts and chondrocytes (Smith and Ma, 2004).

Interestingly, some studies have demonstrated that nanostructured surfaces do not increase adhesion and proliferation uniformly for all cell types. For example, nanostructured surfaces have been demonstrated to be preferred by osteoblasts over other cell types, mainly fibroblasts, which could prevent fibrous encapsulation of a bone implant (Webster *et al*., 2000a,b; Price *et al*., 2004). This is explained by the relatively small and less complicated vtronectin molecule in comparison with other ECM molecules (Webster *et al*., 2000a).

An important finding in studies of cellular responses to nanostructured surfaces is that the dimensions of the topographical features are critical to the cell-surface interactions, and these dimensions are strongly cell-type dependent. Webster *et al*. demonstrated that calvarian rat osteoblast proliferation and production of alkaline phosphatase is significantly higher on a nanostructured surface less than 100 nm in roughness than on a flat surface or on a surface with roughness greater than 100 nm. They found out that the proliferation and production of alkaline phosphatase was significantly higher on materials where the surface roughness ranged between 24-67 nm (Webster *et al*., 2000b). Osteoblast cell line MC3T3-E1 has been found to be sensitive to topographic features on the order of 5 nm (Washburn *et al*., 2004). Blood leukocytes, human microvascular endothelial cell lines, umbilical primary endothelial cells (Buttiglieri *et al*., 2003) and fibroblasts (Dalby *et al*., 2002) show increased adhesion to surfaces with roughness of 13 nm.

A novel, promising and not yet fully explored idea, which we have investigated in this study, is the use of carbon nanoparticles, i.e. fullerenes, nanotubes and nanodiamonds, as components of advanced nanostructured materials for bone tissue engineering.

## 4.2. Fullerenes

### 4.2.1. Characterization of fullerenes

Fullerenes are spheroidal molecules made exclusively of carbon atoms (e.g. $C_{60}$, $C_{70}$), and are present in terrestrial as well as extraterrestial material. Their diameter is about 7.2 Å. Fullerenes were discovered, prepared and systemized by Kroto *et al*. (Kroto *et al*., 1985; Buntar *et al*., 1997). Fullerenes have unique physical and chemical properties which can also be useful in biomedicine. For example, their hollow cage-like shape and structure, similar to clathrin-coated vesicles in cells, can be used in gene delivery (Isobe *et al*., 2006) or drug delivery (Bakry *et al*., 2007). They are not soluble in polar solvents such as methanol or water (Andrievsky *et al*., 1995; Ruoff *et al*., 1993). Fullerenes can form complexes with other atoms and molecules, e.g. metals (Matsuo *et al*., 2008), porphyrins (Bhattacharya *et al*., 2007), nucleic acids (Isobe *et al*., 2006), proteins Belgorodsky *et al*., 2006) or carbon nanotubes (Cheng *et al*., 2007). They can be functionalised with various chemical groups, such as hydroxyl (Guirado-López and Rincón, 2006), carboxyl (Fumelli *et al*., 2000), carbonyl (Huang *et al*., 2006), ester (Burley *et al*., 2002) or aldehydic groups (Cataldo, 2007), which render them soluble in water and enable them to interact with biological systems.

An interesting feature of fullerenes, very important for their biomedical applications, is their potential to generate oxygenated radicals. When irradiated with visible or ultraviolet light, fullerenes can convert molecular oxygen into highly reactive singlet (i.e., atomic) oxygen. Thus, they have the potential to inflict photodynamic damage on biological systems, including damage to cellular membranes as well as various intracellular molecules, including inhibition of various enzymes or DNA cleavage. However, this harmful effect can be advantageously exploited for photodynamic therapy against tumors, viruses and bacteria resistant to multiple drugs. On the other hand, these molecules can also act as the world's most efficient radical scavengers. This is due to the relatively large number of conjugated double bonds in the fullerene molecule, which can be attacked by radical species. Thus, fullerenes would be suitable for applications in quenching oxygen radicals in medicine as well as in cosmetics (for a review, see Bačáková *et al*., 2008).

### 4.2.2. Oxidative effects of fullerenes

#### 4.2.2.1. Inhibition of various enzymes by fullerenes

Fullerenes inhibit the activity of GST and GCL (Usenko *et al*., 2008). Polyhydroxyfullerols were found to inhibit microsomal cytochrome P450-dependent monooxygenases and mitochondrial oxidative phosphorylation (Ueng *et al.*, 1997). Inhibition of these enzymes leads to further enhancement of the oxidative stress generated by fullerenes.

Wolff *et al*. tested the effect of trisamine $C_{60}$-fullerene adducts on neuronal nitric oxide synthase. They reported that $C_3$-trisamine inhibited this enzyme when $IC_{50}$ = 63 nm and $D_3$-trisamine in $IC_{50}$ of 38 nm. This inhibition was fully reversible by calmodulin and skeletal muscle troponin C, but not by skeletal muscle parvalbumin. $C_3$- and $D_3$-semiamine adducts inhibited $Ca^{2+}$-dependent nitric oxide production in $GH_3$ pituitary cells (Wolff *et al*., 2002).

#### 4.2.2.2. Effects on DNA and genotoxicity of fullerenes

Boutorine *et al*. reported that fullerene-ologonucleotide can bind single-stranded DNA, double-stranded DNA, and double-stranded DNA with a hairpin to form a duplex, a triple helix, and a triple helix with a hairpin. In each case the conjugate was cleaved specifically at guanine residues proximal to the fullerene moiety upon exposure to light (Boutorine *et al*., 1994). On the other hand, when the genotoxicity of fullerene and fullerol compounds was studied by assaying mutations in *Escherichia coli* and larvae, it was found that both fullerene and fullerol were essentially non-genotoxic (Jensen *et al.*, 1996).

#### 4.2.2.3. Photodynamic therapy using fullerenes

Photodynamic therapy is an important tool for treating tumors. This therapy is a non-invasive method for treating various types of tumors with reduced side effects (Dima *et al*., 2002; Huang, 2005). Photodynamic therapy has been studied for more than 30 years, and many photosensitizers have been designed for this purpose (Detty *et al*., 2004; Reynolds, 1997). For this therapy, it is neccessary to have a photosensitizing agent with high efficiency of light-induced reactive oxygen species generation. It was mentioned above that fullerenes are able to produce a high amount of reactive oxygen species (ROS) after exposure to weak visible light, and thus could be used as a photosensitizer for photodynamic therapy (Tabata *et al*., 1997; Tsuchiya *et al*., 1996).

It has been reported that polyethylene glycol (PEG) conjugated with $C_{60}$ enhanced the solubility of fullerenes in water and also their accumulation in a tumor (Tabata *et al.*, 1997). Liu *et al*. investigated the antitumor effect of $C_{60}$-PEG complex conjugated to Gd in mice.

This complex was injected intravenously into tumor-bearing mice. After light irradiation, significant tumor photodynamic therapy was observed, though the effect depended on the timing of the light irradiation (Liu *et al*., 2007).

#### 4.2.2.4. Anticancer effects of fullerenes

Polyhydroxyfullerol $C_{60}(OH)_{24}$ was found to inhibit the growth of HEP-2 epidermal carcinoma cells via inhibition of mitotic spindle formation (Simić-Krstić, 1997).

Malonic acid adducts are studied in the biomedical field, not only due to their unique physical and chemical properties, but also because they can easily be prepared in large quantities. These properties include (1) minor modification in the structure of the parent $C_{60}$ cage; (2) good solubility and poor aggregation formation in an aqueous solution when the number of addends is more than one (Guldi and Asmus, 1999); (3) reliable availability of a variety of regioisomers with defined three-dimensional structures (Lamparth and Hirsch, 1994). For these reasons, the malonic acid adducts of $C_{60}$ have become a major object of interest in biomedicine, including anticancer therapy.

Yang *et al*. studied the cytotoxicity effect of three different malonic acid derivates of $C_{60}$ (dimalonic acid $C_{60}$, trimalonic acid $C_{60}$ and quadrimalonic acid $C_{60}$ on human cervix uterus tumor-derived HeLa cells. They found that all three malonic acid $C_{60}$ derivates were cytotoxic toward HeLa cells under light irradiation. The photosensitive cytotoxicity of $C_{60}$ adducts decreased with a rise in the additive number. Thus the greatest cytotoxicity effect was found in the use of dimalonic acid $C_{60}$. This derivate induced a decrease in $G_1$ and a rise in $G_2$ + M during the HeLa cell cycle in the presence of light irradiation (Yang *et al*., 2002).

Fullerenes $C_{60}$ derivatized with nonomalonic acid were also found to inhibit acetylcholine-induced relaxation in the endothelium. This fullerene adduct does not affect receptors mediating contraction and relaxation of muscle (Satoh *et al*., 1997a,b). Lipid peroxidation in the nervous tissue was also inhibited by malonic acid $C_{60}$, which was capable of eliminating both superoxide anions and $H_2O_2$ (Dugan *et al*., 2001).

#### 4.2.2.5. Antiviral and antibacterial properties of fullerenes

Friedman et al. found that a $C_{60}$ molecule can fit into the hydrophobic cavity of HIV protease and inhibit its function (Friedman *et al*., 1993). It was also reported that N-tris(hydroxymethyl)propylamido methanofullerene was active against HIV-1 in acutely infected human peripheral blood mononuclear cells with EC50 = 2.5 μM (Da Ros and Prato, 1999). The effect of fullerenes on viruses has also been studied. Fullerenes have been shown

to inactivate Semliki Forest virus, vesicular stomatitis virus, as well as simian immunodeficiency and Moloney leukemia virus *in vitro* (Käsermann and Kempf, 1997; Nacsa *et al*., 1997). Nonderivatized $C_{60}$ was also found to inhibit the activity of reverse transcriptase with an $IC_{50} \sim 0.3$ μM (Nacsa *et al*., 1997).

Mashino and co-workers studied the antibacterial effect of cationic and anionic fullerene derivates against gram-positive bacteria *in vitro*. They reported that cationic fullerene derivate $C_{60}$-bis(N,N-dimethylpyrrolidinium) iodide inhibited the growth of *Escherichia coli,* but anionic fullerene derivate $C_{60}(C(COOH)_2)_2$ did not. They also found that the antibacterial effect of cationic fullerene derivate was decreased after adding alkyl chains (Mashino *et al*., 2003).

### 4.2.3. Antioxidative properties of fullerenes

#### 4.2.3.1. UV-protective effects of fullerenes

Fullerenes absorb strongly in the ultraviolet regions (UV) and moderately in the visible regions of the spectrum, and form a long-lived triplet state. This property can be used for protection against strong UV irradiation (Aborgast *et al*., 1991). Fullerenes also react rapidly with all sorts of reactive free radicals, and this advantage has been applied in biomedicine (Tebbe *et al*., 1991). This advantage of fullerenes makes them cell-protective against anoikia. Anoikia is cell death induced by growing substrate deprivation. $C_3$-fullero-*tris*-methanodicarboxylic acid was able to induce resistance against anoikia in epithelial A431cells. During cell exposure to UVB, derivates of this fullerene helped to maintain the actin filament distribution in the cell. Exposure to $C_3$-fullero-*tris*-methanodicarboxylic acid alone did not modify the actin network (Straface *et al*., 1999). Carboxyfullerenes were also found to protect human keratinocytes from UVB-induced apoptosis. They significantly reduced the UVB-induced inhibition of keratinocyte proliferation and protected keratinocytes from apoptosis caused by UVB irradiation in a time- and dose-dependent manner. The percentage of cells with depolarized mitochondria was significantly lower in UVB-irradiated keratinocytes treated with carboxyfullerenes. It was found that UVB downregulates bcl-2 in human keratinocytes, and carboxyfullerenes are not able to prevent these effects. It is probable that carboxyfullerenes protect keratinocytes from UVB irradiation through a mechanism interfering with the generation of ROS from depolarized mitochondria without the presence of bcl-2 protein (Fumelli *et al*., 2000). Huang and co-workers investigated the effect of carboxyfullerenes on apoptosis. These compounds were found to prevent TGF-β-induced apoptosis in TGF-β treated human hepatoma HEP-3B cells (Huang *et al*., 1998).

#### 4.2.3.2. Radioprotective effects of fullerenes

Fullerenes have also been shown to be protective against X-rays. Trajković *et al*. studied the radioprotective effect of polyhydroxyfullerols against X-rays (intensity of 8MV) in concentrations of 10 and 100 mg/kg i.p., and compared the effect to the standard radioprotector, amifostine. Both compounds were given to rats 30 min. before irradiation. The radioprotective effect of fullerenol was higher in a dose of 100 mg/kg i.p. (than the effect in a dose of 10 mg/kg p.i.), and was similar to the effect of amifostine (300 mg/kg p.i.). Fullerenol had a better radioprotective effect on the spleen, small intestine and lung, while amifostine had a better protective effect on the heart, liver and kidney (Trajković *et al*., 2007).

#### 4.2.3.3. Protective effects of fullerenes against oxidizing chemicals

The protective effect of cystine $C_{60}$ derivative on hydrogen peroxide-induced apoptosis in rat pheochromocytoma PC12 cells was investigated by Hu *et al*. From their studies it is known that cystine is water soluble and is required for maintaining cellular levels of glutathione, which is essential in cell protection against oxidative stress and various toxins. Cystine $C_{60}$ was found to penetrate through the cell membrane and reduce the accummulation of ROS and cellular damage caused by hydrogen peroxide (Hu *et al*., 2007).

Monti *et al.* presented the protective activity of carboxyfullerene against oxidative stress–induced apoptosis in human peripheral blood mononuclear cells (PBMCs). Apoptosis was induced with 2-deoxy-D-ribose (dRib) or TNF-α with cycloheximide, and the result was production of ROS. Carboxyfullerene derivates were able to significantly inhibit apoptosis in both cases, but they were slightly more effective in preventing mitochondrial membrane depolarisation induced by TNF- α than by dRib (Monti *et al*., 2000).

#### 4.2.3.4. Neuroprotective effects of fullerenes

Since fullerenes are well known as an effective "radical sponge", they have been considered for wide use in neurology and neuropathology. The reason is that many neurodegenerative disorders, such as Parkinson's, Alzheimer's and Lue Gehrig's are accompanied by massive production of oxygen and nitric oxide radical species, probably due to over-excitation of the glutamic acid receptors (Bosi *et al*., 2003). It has been reported that polyhydroxyfullerols decreased excitotoxic neuronal death by 80% following N-methyl-D-aspartate (NMDA) treatment (Dugan *et al*., 1996). Dugan *et al*. also reported that systemic administration of $C_3$ carboxyfullerene isomer delayed motor deterioration and death in a mouse from familial amyotrophic sclerosis (Dugan *et al*., 1997). It was found that microinjection of fullerenes in

complex with polyvinylpyrrolidone into the hippocampus prevented amnesia elicited by protein synthesis blockade in rats (Podolski *et al*., 2004). Podolski *et al*. also reported that intracerebroventricular injection of hydrated $C_{60}$ fullerenes prevented impairment of performance of a cognitive task induced by amyloid-$\beta_{25-35}$, and also inhibited Aβ fibrillization *in vitro* (Podolski *et al*., 2007).

Tykhomyrov *et al*. demonstrated that hydrated fullerenes in a concentration of 30 nM protect the central nervous system in rats from damage caused by oxidative stress. Hydrated fullerenes were applied to rats during chronic alcoholization, and were found to prevent pathological loss of astrocytes (i.e., the main supportive cell type of CNS), as well as the astrocytic marker, glial fibrillary acidic protein. Due to their adaptogenic effects, hydrated fullerenes significantly improve the behavioral response and eliminate the emotional deficits induced by chronic alcohol uptake (Tykhomyrov *et al*., 2008).

#### 4.2.3.5. Antiallergenic effects of fullerenes

Another consequence of the antioxidative propereties of fullerenes is inhibition of allergic responses, e.g. by reductions in activation of the signaling molecules involved in oxidative stress. For example, human mast cells (MC) and peripheral blood basophils are critical cells involved in the initiation and propagation of several inflammatory condititions, mainly type I hypersensitivity. Water soluble fullerene derivatives polyhydroxy $C_{60}$ and N-ethyl-polyamino $C_{60}$ were shown to be negative regulators of allergic mediator release that suppress Ag-driven type I hypersensitivity. Human MC and peripheral blood basophils exhibited significant inhibition of IgE dependent mediator release when preincubated with polyhydroxy $C_{60}$ and N-ethyl-polyamino $C_{60}$. Fullerenes also prevented the *in vivo* release of histamine and a drop in core body temperature in vivo using an MC-dependent model of anaphylaxis. This knowledge can lead to a new way for controlling MC-dependent diseases such as asthma, inflammatory arthritis, heart diseases or multiple sclerosis (Ryan *et al*., 2007).

### 4.2.4. Fullerenes in tissue development, regeneration and tissue engineering

Tsuchiya *et al*. reported that fullerenes solubilized with polyvinylpyrrolidone promoted chondrogenesis and differentiation of LB and B lymphoblast line cells in a concentration-dependent process (Tsuchiya *et al*., 1995).

Some studies reported that fullerenes also appeared suitable for treating tissues, e.g. cartilage tissue. Kurz *et al*. demonstrated that the extent of mechanical stress on articular cartilage stimulates excess production of ROS from chondrocytes, leading to

depolymerization of hyaluronic acid and chondrocyte death, which could be prevented by fullerenes (Kurz *et al*., 2004, 2005).

Yudoh *et al*. reported that water soluble fullerenes $C_{60}$ prevent the degeneration of articular cartilage in osteoarthritis. It is known that catabolic-stressed chondrocytes produce excess amounts of ROS as well as proinflammatory chemokines. Water soluble fullerenes (100 μM) are known to inhibit the catabolic stress-induced production of matrix-degrading enzymes (matrix metalloproteinases 1,3 and 13), down-regulation of matrix production, apoptosis and premature senescence in human chondrocytes *in vitro*. The inhibitory effect was dose dependent. Water soluble fullerenes in combination with hyaluronate (HA) have caused a significant reduction in cartilage degeneration compared with treatment with fullerene or HA alone (Yudoh *et al*., 2007).

Fullerenes also have affinity with the bone tissue and can affect bone tissue mineralization. González and co-workers investigated the effect of a tissue-vectored bisphosphonate fullerene $C_{60}(OH)_{16}$AMBP [4,4-bisphosphono-2-(polyhydroxyl-1,2-dihydro-1,2-methanofullerene[60]-61-carboxamido)butyric acid] on bone mineralization. Hydroxyapatite (HAP) is a primary inorganic component of bone. This molecule offers many binding sites for structurally suitable molecules. $C_{60}(OH)_{16}$AMBP was found to have strong affinity for bonding to HAP. When 1 μM $C_{60}(OH)_{16}$AMBP was used, hydroxyapatite mineralization was reduced by 50%. This knowledge can be useful for example in bone radiation therapy, where the radionuclide-containing fullerene can be bound to the diseased site in the bone. This kind of therapy can reduce the dose level required by the subject and avoid damage to non-diseased tissue (González *et al*., 2002).

An important issue is that relatively little is known about the effects of fullerenes on cell-substrate adhesion, i.e., when fullerenes are deposited on the material surface in the form of thin layers. These layers may promote adhesion, growth and further functions of cells because of their nanostructure. In addition, these layers could be deposited through a mask in the form of micropatterned layers, which could induce regionally-selective cell adhesion and directed cell growth. Last but not least, fullerene molecules can be combined with metals, e.g. titanium, which is currently used for constructing bone and joint replacements in clinical practice.

## 4.3. Carbon nanotubes

### 4.3.1. Characterization of carbon nanotubes

Carbon nanotubes (CNTs) are members of the fullerene structural family. Whereas fullerenes (buckyballs) are spherical in shape, nanotubes are cylindrical. Thus, their name is derived from their shape. The diameter of carbon nanotubes is only one to a few nanometers, while they can be several millimeters or even centimeters in length.

There are two main types of carbon nanotubes: single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs). SWNTs are formed by one cylindrical graphene sheet, whereas MWNTs contain two or more concentrically arranged graphene sheets. Multi-walled nanotubes can be considered as a collection of concentric SWNTs with different diameters, and thus their properties are quite different from the properties of SWNTs (Dresselhaus *et al*., 1996; Iijima, 1991). Similarly to graphite, the carbon atoms in the nanotubes are associated by $sp^2$ bonds. These bonds are stronger than $sp^3$ found in diamond. Thus, CNTs are one of the strongest and stiffest materials known. The tensile strength of MWNTs ranges between 11-63 GPa, while the tensile strength of steel is about 1.2 GPa (Yu *et al.*, 2000).

### 4.3.2. Preparation of carbon nanotubes

Carbon nanotubes are generally produced by three main techniques, namely arc discharge (Anazawa *et al*., 2000; Lee *et al*., 2002), laser ablation (Maser *et al*., 1998; Scott *et al*., 2001) and chemical vapour deposition (CVD) (Yudasaka *et al*., 1995). The preparation of SWNTs requires metal catalysts such as Co, Ni, Fe or Y, while MWNTs do not require these catalysts. The ***Arc discharge*** method is the commonest and easiest way to produce carbon nanotubes. A high temperature (>3000°C) is needed to evaporate the carbon atoms into a plasma to form both single-walled and multi-walled nanotubes. This technique produces a mixture of components and requires separation of the nanotubes from the soot and the metallic catalyst. This method creates nanotubes through arc-vaporisation of two carbon rods placed end to end, separated by approximately 1mm, in an enclosure that is usually filled with an inert gas (helium, argon) at low pressure (between 50 and 700 mbar). Recent investigations have shown that it is also possible to create nanotubes with the arc method in liquid nitrogen (Jung *et al*., 2003). ***Laser ablation*** vaporises graphite in an electrical furnace (filled with helium or argon gas) heated at 1200°C. The purity of the graphite ensures high purity of the carbon nanotubes (Zeng *et al*., 2006). ***Chemical vapour deposition (CVD)*** synthesis is achieved by putting a carbon source in the gas phase and using an energy source, such as plasma or a resistively heated coil, to transfer energy to a gaseous carbon molecule.

Commonly used gaseous carbon sources include methane, carbon monoxide and acetylene. The energy source is used to "crack" the molecule into reactive atomic carbon (Ren *et al*., 1998, 1999). For biomedical purposes, carbon nanotubes need to be purified from catalysts to avoid cytoxicity and potential immunological reactions (Polizu *et al.*, 2006).

### 4.3.3. Purification of carbon nanotubes

There are many ways of purifying CNTs, such as oxidation (Borowiak-Palen *et al*., 2002), annealing (Chiang *et al*., 2001), micro-filtration (Bandow *et al*., 1997), ultrasonication (Hou *et al*., 2001), magnetic purification (Thiên-Nga *et al*., 2002), cutting (chemical or mechanical) (Farkas *et al*., 2002) or functionalisation with soluble groups (Niyogi *et al.*, 2001; Zhao *et al*., 2001). The importance of purity and functionalization of carbon nanotubes in biomedicine was shown by Nimmagadda and co-workers. They investigated the growth and viability of 3T3 fibroblasts on three different types of SWNTs: non-purified, purified and functionalized with glucosamine. It was found that the cytotoxicity of carbon nanotubes is dose-dependent, and functionalization improves the biocompatibility of CNTs. The viability of 3T3 fibroblasts decreased to 55% after 3 days when cultivated on the lowest concentration (0.001% wt/vol) of apurified SWNTs, and a progressive increase in redox potential was seen. At concentrations of 0.0625% wt/vol, no cellular metabolic activity was found, which indicated death of the cells. A significant increase in viability was found when 3T3 fibroblasts were incubated with purified or functionalized SWNTs in comparison with apurified SWNTs at concentrations lower than 0.5% wt/vol. 3T3 fibroblasts incubated with functionalized SWNTs showed significantly higher viability than cells cultured at the same concentration of purified SWNTs (Nimmagadda *et al*., 2006).

### 4.3.4. Potential cytotoxicity of carbon nanotubes

As mentioned above, for the application of carbon nanotubes in biomedicine, it is necessary to know whether they are cytotoxic. In addition to the presence of impurities, e.g. the catalysts mentioned above, the mechanism of the cytotoxic effects of CNTs leads to the oxidative damage described for fullerenes, and is also dependent on nanotube size, functionalization, water solubility and formation of agglomerates (Bačáková *et al.*, 2008,). A number of publications have reported no apparent cytotoxicity (Chen *et al*., 2006; Dumortier *et al*., 2006; Yehia *et al*., 2007; Zhu *et al*., 2006), while others have reported varying degrees of significant cytotoxicity (Lam *et al*., 2003; Monteiro-Riviere *et al*., 2004; Wick *et al*., 2006). Yehia and co-workers studied the cytotoxixity of SWNTs dispersed in Dulbecco's modified

Eagle medium (DMEM) on HeLa cells. They found that the uptake of SWNTs by HeLa cells was a time- and temperature-dependent process (Yehia *et al*., 2007). The fetal bovine serum present in DMEM contains a mixture of various dissolved substances such as proteins, lipids, steroid hormones, minerals and metabolites. The most notable serum components known to solubilize CNTs are bovine serum albumin (BSA) and phospholipids (Karajanagi *et al*., 2006; Lin *et al*., 2004). These proteins facilitate uptake of CNTs by cells. Bianco *et al*. reported that CNT follows a temperature- and endocytosis-independent mechanism (Bianco *et al*., 2005a,b; Pantarotto *et al*., 2004).

Heller and co-workers found an accumulation of DNA-coated SWNTs in the perinuclear zone of 3T3 cells, but not in the nuclear envelope (Heller *et al*., 2005). Lam *et al*. investigated the pulmonary toxicity of CNTs in mice. B6C3F1 mice were intratracheally instilled once with 0.1 or 0.5 mg of CNTs suspened and ultrasonicated in 50µl of mouse serum. CNTs with different amounts of metal (27% and 2% w/w of iron and CNTs containing 26% of nickel and 5% of yttrium) induced dose-dependent formation of epithelioid granulomas in the centrilobular alveolar septa and, in some cases, interstitial inflammation (Lam *et al*., 2004). Shvedova and co-workers reported that exposure of keratinocytes to SWNTs caused free radical generation, accumulation of peroxidative products, antioxidant depletion, and loss of cell viability. Exposure to SWNTs also caused ultrastructural and morphological changes in keratinocytes (Shvedova *et al*., 2003).

Wick *et al*. found that carbon nanotube cytotoxicity is also dependent on the degree and kind of agglomeration. Four different fractions of SWNTs were studied: CNT raw material, CNT agglomerates, CNT bundles and CNT pellets. All CNT fractions, except well dispersed CNT bundles, were aggregated after the incubation time to micron-sized structures. CNT raw material significantly decreased the cell activity and proliferation in a dose-dependent way. The CNT agglomerates were the most toxic fraction. After 3 days of incubation, treatment with CNT agglomerates evoked a round-shaped cell morphology of the MSTO-211H cells, similar to cells treated with asbestos. In contrast, CNT bundles did not induce any visible changes, and no agglomerates were formed during the incubation time. A comparison of CNT agglomerates with CNT bundles containing similar impurities clearly showed the importance of dispersion CNTs used for biomedical applications (Wick *et al*., 2007).

### 4.3.5. Biomedical applications of carbon nanotubes, and their future uses in tissue engineering

The chemistry of carbon nanotubes, lying in their derivatization and purification state, is essential in their development as biomaterials (Polizu *et al*., 2006). Carbon nanotubes have been studied in biomedicine as building blocks for constructing nanoelectrodes and nanosensors, cathodes for imaging X-ray radiation, nanotweezers for biological applications, nanopipettes and components for medical robots.

The unique properties of carbon nanotubes also make them promising for tissue engineering applications (Polizu *et al*., 2006), i.e., in the form of CNT layers, meshes or composites of CNTs with natural and synthetic polymers. Zanello *et al*. successfully used layers of pristine CNTs or CNTs functionalized with positively or negatively charged chemical groups for cultivating osteosarcoma ROS17/2.8 cells (Zanello *et al.*, 2006).

Carbon nanotube substrates have also been found to be excellent substrates in regeneration of neural tissue *in vitro*. Because carbon nanotubes are non-biodegradable, these nanoparticles can be used as implants where long-term extracellular molecular cues for neurite outgrowths are necessary. Matsumoto *et al*. investigated the growth of neurons on neurotrophin-coated MWNTs. These materials have been shown to stimulate the neurite outgrowths of neurons, and no cytotoxicity was present (Matsumoto *et al*., 2006). Mattson *et al.* studied the growth of rat embryonic hippocampal neurons on MWNTs unmodified and modified with 4-hydroxynonenal (4-HNE). Neurons grown on nanotubes modified with 4-HNE had more elaborated neuritic arbors than did neurons grown on unmodified MWNTs (Mattson *et al*., 2000). In addition to neuronal regeneration, there are studies which support the application of CNT in bone, cartilage and vascular tissue engineering.

Abarrategi and co-workers investigated cells of mouse myoblast cell line C2C12 in cultures on composites of MWNTs with chitosan (i.e., a natural degradable polymer known for its good biocompatibility) and adsorbed with a recombinant human bone morphogenetic protein-2 (rhBMP-2), which has an osteoinductive effect. This material seemed to be suitable for cell adhesion, spreading, proliferation and, in addition, C2C12 cells underwent osteoblastic differentiation. The same scaffolds were also investigated *in vivo* in mouse. MWNT-chitosan with adsorbed rhBMP-2 was implanted for 3 weeks into a subcutaneous pocket made in the mouse back muscle tissue. Bone tissue regeneration was observed with significant scaffold structure degradation and there was no inflammation reaction (Abarrategi *et al*., 2008). Composites of CNTs with inorganic components of bone tissue matrix, such as hydroxyapatite, were also investigated. Similarly, as mentioned above (for more details see

chapter 2.4.), hydroxyapatite markedly supports osteoblast growth. Balani *et al*. studied human osteoblasts on plasma-sprayed carbon nanotube-reinforced hydroxyapatite coatings, and found that the hFOB progenitor cells were spread very well and had differentiated to mature cells (Balani *et al.*, 2007).

The use of CNT in vascular tissue engineering is supported by studies performed by MacDonald *et al.* They reported that rat aortic smooth muscle cells (RASMCs) cultivated on similar collagen-SWNT composites, containing various concentrations of SWNTs (0.2, 0.4, 0.8 and 2.0% wt/vol), showed similar viability on all tested composites comparable with control (MacDonald *et al*., 2005).

The electrical properties of CNTs enable electrical stimulation of cells, which has repeatedly been shown to promote cell adhesion, growth, maturation and function. For example, chondrocyte adhesion was more than 50% higher on electrically stimulated MWNT-polycarbonate urethane (PCU) composite compared to non-electrically stimulated composite and bare PCU (Khang *et al*., 2008). In addition, electrically-stimulated osteoblasts growing on composites of carbon nanotubes and polylactic acid increased their proliferation and differentiation activity, their production of mineralized bone matrix and thus healing of the damaged bone tissue (Supronowicz *et al*. 2002).

An important finding is that CNTs can be cleared from the organism by glomerular filtration (McDevitt *et al.*, 2007; for a review, see Bačáková *et al*., 2008). This finding markedly increases the chance of nanotubes being used as components of degradable scaffolds for tissue engineering (Abarrategi *et al*., 2008; MacDonald *et al*., 2005). Another possibility, explored in our studies and presented in this thesis, is to mix carbon nanotubes with biostable non-degradable polymers, such as a terpolymer of polytetrafuoroethylene (PTFE), polypropylene (PP) and polyvinyldifluoride (PVDF) (Bačáková *et al*., 2007). Composites of nanotubes with polycarbonate urethane (PCU, (Khang *et al*., 2008) have also been studied for biomedical applications.

## 4.4. Nanocrystalline diamond

### 4.4.1. Characterization and preparation of nanocrystalline diamond

In recent years, nanocrystalline diamond (NCD) has become widely investigated for its remarkable properties, such as high hardness and wear resistance, low friction coeficient (Elam, 2004; Tjong and Chen, 2004), excellent biocompatibility (Amaral *et al*., 2008; Bačáková, 2007; Grausová *et al.*, 2008a,b,c), and good electrical properties (Corrigan *et al*., 2002; Krauss *et al*., 2001). These unique properties enable the application of NCD not only in

electronics and optics, but also in biology and medicine. NCD can be prepared in the form of a powder by detonation synthesis from a mixture of trinitrotoluene and hexogene (Iakoubovskii *et al*., 1999), and thin NCD films can be deposited by microwave plasma-enhanced chemical vapour deposition (MW PECVD) from methane or fullerene precursors (Quint *et al*., 1998).

### 4.4.2. Solubilization of nanocrystalline diamond

For the application of diamond nanoparticles in biomedicine, their solubility in aqueous systems, including buffer solutions, is important. Nanodiamond (especially the material from detonation synthesis) exists in the form of strongly bound agglomerates, due to the harsh conditions in the reaction chamber (Aleksenskii *et al*., 2000). Diamond nanoparticles are bound not only by electrostatic interactions, but also through covalent bonds between the surface functional groups and by soot structures surrounding each nanoparticle. Stable colloidal systems can be obtained in polar, protic solvents, for example water, dimethylsulfoxide (DMSO) or methanol (Krueger, 2008). Xu *et al*. reported that sodium oleate can be used as a detergent to disperse nanodiamond clusters (Xu *et al*., 2005). The bead-assisted sonic disintegration technique (BASD) combines the shear force induced by zirconia beads with the cavitation produced by ultrasound, and proved very efficient for the deagglomeration and *in situ* functionalisation of small quantities of diamond. The stability of the colloidal solutions from BASD depends on the polarity and hydrogen bonding ability of the solvent, with best results obtained for DMSO and water (Krueger, 2008; Ozawa *et al*., 2007).

### 4.4.3. Immobilization and detection of biomolecules using nanocrystalline diamond

The bare diamond surface has proven to be very reactive toward adsorption of various kinds of small and larger molecules. Larger organic molecules and biological structures can be immobilised on the diamond surface (Christiaens *et al*., 2006; Härtl *et al*., 2004). For example, Bondar et al. showed the adsorption of apoobelin and luciferase (Bondar *et al*., 2004), Huang and Chang reported on the immobilisation of cytochrome c (Huang and Chang, 2004), and Chung on the application of protein lysozyme for surface modification of diamond particles (Chung *et al*., 2006; Nguyen *et al*., 2007). The binding strength depends strongly on the surface termination of the diamond material. Hydrogen-terminated surfaces have low affinity for protein bonding due to their hydrophobicity (Tryk *et al*., 2007).

The affinity of biological molecules to nanodiamond particles and films can be utilized for detecting, separating and purifying these molecules. Nanodiamond has been used as an adsorbent for large biomolecules such as proteins, which can be useful for detecting them in dilute solutions by MALDI-TOF mass spectrometry (Kong *et al*., 2005a). This method can also be used for detecting DNA oligonucleotide after coating with poly-L-lysine (Kong *et al*., 2005b). Adsorption of molecules on a nanodiamond surface has been shown for protein purification and separation (Bondar *et al*., 2004) and for immobilisation of antibodies for sensor application. Huang and co-workers have described a system for detecting *Salmonella typhimurium* and *Staphylococcus aureus* (Huang *et al*., 2004). Kossovsky *et al*. used cellobiose-coated diamond nanoparticles for immobilising a mollusc-derived molecule, mussel adhesive protein (MAP), to generate antibodies in rabbits (Kossovsky *et al.*, 1995).

### 4.4.4. Nanocrystalline diamond in bioimaging

Yu *et al.* reported that nanodiamond particles can be used as fluorescence labels because of their intrinsic capability to emit light. They have studied proton-irradiated and annealed nitrogen-containing nanodiamonds in kidney, and have found that besides strong luminescence in the cells, they did not observe any photobleaching or cytotoxicity (Yu *et al.*, 2005). Similarly, Fu and co-workers demonstrated that nanodiamond is a promising biomarker candidate for *in vivo* imaging and diagnosis. After incubating HeLa cells with NCD (35 nm in size), intense cell autofluorescence was observed at 510-560 nm when exposed to blue light at 476 nm. They also reported that under the same exctitation conditions the fluorescence of a single 35 nm diamond is significantly brighter than that of a single dye molecule, such as Alexa Fluor 546 (Fu *et al.*, 2007).

### 4.4.5. Non-toxicity of nanocrystalline diamond

An important feature of nanodiamonds is that in comparison with fullerenes and nanotubes, these carbon nanoparticles are practically lacking in cytotoxicity. The non-cytotoxic effect of diamond nanoparticles has been reported by Schrand *et al*. in experiments with macrophages and neuroblastoma cells. Nanodiamonds did not produce significant ROS, and cells were grown on this material without morphological changes compared to control (Schrand *et al*., 2007). Aspenberg *et al.* investigated microdiamond particles mixed with hyaluronan *in vivo* in rabbits, and found that diamond particles are harmless and do not cause a decrease in bone formation (Aspenberg *et al*., 1996).

On the other hand, while many scientists report harmless effects of diamond nanoparticles, Puzyr and co-workers found that nanodiamonds in colloid form cause destruction of white cells and erythrocytes. It was shown that the destruction of white cells in the presence of NCD was 85-93% after 90 min. of incubation, while the destruction of white cells in a medium without NCD was only 12-16%. The 3-50% erythrocytes hemolyzed within 90 min. after adding NCD in the final concentration of 0.031 wt%. They presumed that the determinative factor is the ability of NCD to adsorb protein molecules, and binding of NCDs with the cell membrane of blood cells probably causes irreversible damage in their structure, leading to hemolysis (Puzyr *et al*., 2004). With the exception of this finding, nanodiamonds have been found to be non-cytotoxic in many studies.

### 4.4.6. Non-immunogenicity of nanocrystalline diamond

Several studies have also shown non-immunogenic properties of diamond nanoparticles. Tang and co-workers have investigated the adhesion of polymorphonuclear leucocytes (PMN) to a nanodiamond surface *in vitro* as well as *in vivo*. PMN are cells involved in inflammatory responses. For example, after material implantation they can accumulate on this material, leading to its degradation. For a successful material implantation, the accumulation of polymorphonuclear leucocytes should be reduced to a minimum. The adherence of PMN to an NCD surface was as low as on commercially used 316 steel and 40% lower than on titanium. *In vivo* experiments have also shown minimal inflammatory responses and tissue disturbance, e.g., surrounding fibrosis (Tang *et al*., 1995).

Nordsletten *et al*. reported that NCD does not evoke an inflammation reaction in experiments with human monocytes, other cells involved in an inflammatory response. They compared the adhesion and production of interleukin-1 by monocytes on diamond microparticles to HAP and silicon carbide. Interleukine-1 is a proinflammatory cytokine produced by various cells involved in inflammatory rections such as monocytes, lymphocytes, macrophages, endothelial cells or fibroblasts. Monocytes adhered to diamond particles were round and small in size, and the production of IL-1was very low and often undetectable. On the other hand, monocytes on hydroxyapatite and silicone carbide were elongated and spindle-shaped, and their production of IL-1 increased significantly (Nordsletten *et al*., 1996). Knowledge that diamond is non-cytotoxic and does not evoke inflammation enables this material to be successfully used in tissue regeneration.

### 4.4.7. Nanodiamond films as biomaterial coatings and cell carriers for tissue engineering

Due to their extreme hardness and excellent tribological characteristics, diamond films have been investigated for their potential use as biomaterial coatings, e.g. for coating the heads and cups of artificial joints, namely the temporomandibular joint (Papo *et al.* 2004). In addition, they may enhance the bioactivity of the coated materials. It has been reported that ultra-nanocrystalline diamond (UNCD) is a promising candidate for use as an encapsulating coating for implantable retinal microelectronic devices, due to its excellent biocompatibility with the eye (Xiao *et al*., 2006). Bajaj *et al*. investigated the biocompatibility of UNCD films (deposited on silicon wafers) with three different cell types, i.e., cervical carcinoma HeLa cells, pheochromocytoma PC12 and osteoblast MC3T3 cells. The results have shown that UNCD supports adhesion, growth and proliferation in all of the three tested cell types (Bajaj *et al*., 2007).

Nanodiamond coatings have also been investigated in bone tissue engineering, and several studies have reported positive effects of this material on bone regeneration. Amaral *et al*. reported that human MG 63 osteoblast-like cells and human bone marrow cells on NCD coatings were well-attached and completely spread, displaying a flat configuration and a typical morphology. The alkaline phosphatase (ALP) activity, i.e., a marker of cell differentiation toward osteoblastic phenotype, of bone marrow cells on NCD was higher than on the control cells on standard culture dishes. Cell growth was accompanied by the production of fibrillar matrix, and 21-day-old cultures contained mineralized globular structures associated with the fibrous cell layer (Amaral *et al*., 2008).

The bone tissue formation on NCD films can be further improved by immobilising the bone morphogenetic protein-2 (BMP-2) on these layers. BMP-2 was found to bind oxygen-terminated NCD in a highly stable, non-covalent manner, a procedure known as physisorption. It was reported that an O-NCD/ BMP-2 implant used in *in vivo* supports promoted *de novo* bone formation at the implant surface (Kloss *et al*., 2008).

Nanocrystalline diamond films could be also applied for coating the bone-anchoring stems of articular prostheses, or other permanent bone implants, in order to improve their integration with the surrounding bone tissue. Therefore, in our studies (Bačáková *et al*., 2007; Grausová *et al*. 2008a,b,c), we investigated the adhesion, growth, metabolic activity, viability and osteoblastic differentiation of human osteoblast-like MG 63 cells on nanocrystalline NCD films with surface nanoroughness (*rms* of 8.2 nm), as well as hierarchically-organized submicron-sized and nano-sized surface roughness (*rms* of 301 nm and 7.6 nm). The latter

films were chosen, because a hierarchical organization on multiple scales is the main principle for building natural tissues and organs, including bone (Tan and Saltzmann 2003).

## 5. OBJECTIVES OF THE STUDY

The leading hypothesis of this study was that carbon nanoparticles could be successfully used for the construction of bone implants in the form of hard biocompatible layers or powders admixed into a polymeric matrix. The first prerequisite for integration of the bone implants with the surrounding bone tissue, their durability and good function, is their attractiveness for colonization with bone cells. We expected that this colonization would be enhanced on the nanostructured substrates created in this study, due to their similarity with the nanoarchitecture of the natural extracellular matrix. We therefore studied the attachment, spreading, proliferation, viability, metabolic activity, osteogenic differentiation and potential immune activation of human osteoblast-like MG 63 cells in cultures on the following nanostructured carbon-based materials:

- fullerene $C_{60}$ and hybrid titanium-fullerene $C_{60}$ films with a continuous and micropatterned morphology
- composites of single-walled or multi-walled carbon nanotubes with a terpolymer of polytetrafluorethylene, polyvinyldifluoride and polypropylene (PTFE/PVDF/PP)
- nanocrystalline diamond films with purely nanostructured or hierarchically micro- and nanostructured surfaces

## 6. MATERIAL AND METHODS

This thesis is based on nine papers by the author, published or accepted in impacted international journals, one book chapter and one patent application. The materials and methods used for accomplishing the thesis are therefore decribed here only in an abbreviated form, and the details are provided in the attached author's publications. In addition, this study has been highly interdisciplinary, requiring broad collaboration with several chemical and physical institutions, where the artificial substrates for bone cell growth were prepared and characterized.

The continuous and micropatterned fullerene layers and also the hybrid titanium-fullerene layers were prepared at the Institute of Nuclear Physics, Acad. Sci CR, Řež near Prague, by Jiří Vacík, PhD. Briefly, these layers were deposited on carbon fibre-reinforced carbon composites (CFRC) or microscopic glass coverslips in a UNIVEX vacuum reactor.

Their physical and chemical properties were characterized by Raman spectroscopy at the Institute of Physics by Vladimír Vorlíček, PhD., and by atomic force microscopy, reflection goniometry and other methods at the Institute of Chemical Technology by Prof. Václav Švorčík. For more details, see the following papers attached to this work: Bačáková *et al*., 2007; Bačáková *et al*., 2008; Grausová *et al*., 2008 a,b and Vandrovcová *et al*., 2008.

The composites of single-walled or multi-walled carbon nanotubes with the PTFE/PVDF/PP terpolymer were prepared and characterized at the Department of Biomaterials, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow, Poland, in cooperation with Aneta Fraczek, PhD., Ewa Stodolak, PhD., Prof. Stanislaw and Prof. Marta Blazewicz. In addition, degradable scaffolds made of a copolymer of lactic and glycolic acid for potential reinforcement with carbon nanotubes were prepared (Elzbieta Pamula, PhD., from AGH University of Science and Technology, Krakow, Poland). For more details, see the following papers: Bačáková *et al*., 2007; Bačáková *et al*., 2008 and Pamula *et al*., 2008.

The nanocrystalline diamond films were prepared and characterized at the Institute of Physics, Acad. Sci. CR by Alexander Kromka, PhD., Štěpán Potocký, PhD., Prof. Milan Vaněček and Bohuslav Rezek, PhD. The nanostructured or hierarchically micro- and nanostructured layers were deposited on silicon substrates by a microwave PECVD method in an ellipsoidal cavity reactor. For more details, see the attached studies by Bačáková *et al*., 2007; Grausová *et al*., 2008 c, d, e; Bačáková *et al*., 2008.

All biological experiments investigating the adhesion, growth, viability, metabolic activity, differentiation and immune activation of human osteoblast-like MG 63 cells and bovine pulmonary artery endothelial CPAE cells were performed by Lubica Grausová at the Department of Cell Growth and Differentiation, Inst. Physiol., Acad. Sci. CR, and are the main topic of this study.

The markers of adhesion were the number of initially adhering cells 24 hours after seeding, the size of the cell spreading area, the number, morphology, localization and chemical composition of focal adhesion plaques, studied by immunofluorescence of various types of integrin adhesion molecules and integrin-associated proteins, such as talin and vinculin. The concentration of all these molecules was studied semiquantitavely per mg of protein in cell homogenates, using the ELISA enzyme-linked immunosorbent assay. The beta-actin cytoskeleton, which plays an important role in cell spreading, was also studied by immunofluorescence and ELISA.

Cell proliferation was evaluated by changes in the cell number in several time intervals, by constructing growth curves and calculating the cell population doubling time.

The metabolic activity of cells was assessed by the XTT test. The principle of this test is that the metabolically active cells convert a tetrazolium salt XTT into an orange formazan dye by their mitochondrial dehydrogenases.

Cell viability was evaluated using a commercially available LIVE/DEAD viability/cytotoxicity kit, based on staining the live cells with calcein AM so that they emitted a green fluorescence, and staining the dead cells with ethidium bromide to produce a red fluorescence.

Cell differentiation (maturation) was estimated by the presence and content of cell type-specific or typical molecules, such as osteocalcin or osteopontin in osteoblast-like cells or von Willebrand factor in vascular endothelial cells. The formation of a beta-actin cytoskeleton and the concentration of beta-actin per mg of protein were also considered as certain markers of cell maturation in both cell types, especially in the endothelial cells.

The concentration of the intracellular adhesion molecule 1 (ICAM-1), i.e. an immunoglobulin molecule capable of binding the inflammatory cells, was used as a marker of cell immune activation.

# 7. RESULTS AND DISCUSSION

In general, our studies presented in this thesis have shown that all investigated materials, i.e. fullerene-based layers, carbon nanotube-terpolymer composites and nanodiamond layers gave good support for the adhesion, proliferation, viability, metabolic activity and osteogenic differentiation of human bone-derived MG 63 cells, though this supportive effect had some different aspects in the three studied material types.

## 7.1. Interaction of cells with fullerene $C_{60}$ layers

### 7.1.1. Continuous fullerene $C_{60}$ films

In the first study included in this thesis (Bačáková *et al.*, 2007), we investigated the behaviour of MG 63 cells on continuous fullerene coatings deposited on the surface of carbon fiber-reinforced carbon (CFRC) composites. These composites have been considered as promising for implantation into the bone because of their appropriate mechanical properties. However, they are prone to release carbon particles, thus it is always reasonable to coat them with a protective biocompatible layer, such as pyrolytic carbon (Starý *et al.*, 2003a, b), a

carbon-titanium layer (Bačáková *et al*., 2001a) or fullerite, tested in this study. Surprisingly, the number of initially adhering cells on CFRC composites covered with a fullerene layer was significantly lower in comparison with uncoated CFRCs and tissue culture polystyrene dishes, used as control samples. The relatively low cell population density on these fullerene layers may be due to their high hydrophobicity (water drop contact angle about $100^{o}$). This hydrophobia was combined with another factor which could promote a less suitable action for cell adhesion, namely surface microroughness, caused by the prominence of the carbon fibres over the carbon matrix in the CFRC composites (Bačáková *et al.*, 2001a). Thus, the hydrophobia and the surface microroughness of the CFRCs could act synergetically on decreasing cell adhesion. In accordance with this idea, on continuous fullerene layers deposited on microscopic glass coverslips, the population density of MG 63 cells was comparable to the values found on control culture substrates, such as standard polystyrene dishes and uncoated glass coverslips (Bačáková *et al*., 2008; Grausová *et al*., 2008a, b; Vandrovcová *et al*., 2008).

On the other hand, the spreading area of MG 63 cells on the fullerene-coated CFRC composites was significantly larger than on the uncoated CFRC and control. This may be due to the low cell density on the fullerene layer, which provided enough space for cell spreading. On the other hand, cell spreading may be facilitated by the nanoroughness and nanotopography of the fullerene layer. The nanostructure of the material surface is known to enhance the adhesion of cells by improving the spectrum and spatial orientation of the adsorbed cell adhesion-mediating ECM molecules (Webster *et al*., 2000a, b). In accordance with this, MG 63 cells cultivated on fullerene-coated CFRC composites formed well-developed vinculin-containing focal adhesion plaques and beta-actin microfilaments, and thus the cells were viable and were effectively anchored to the fullerene surface (Bačáková *et al*., 2007; Bačáková *et al*., 2008). Similarly, MG 63 cells on fullerene $C_{60}$ layers deposited on microscopic glass coverslips assembled focal adhesion plaques containing $\beta_1$-integrins and talin, associated with a beta-actin cytoskeleton (Grausová *et al*., 2008b). Moreover, these cells exhibited bright fluorescence after immunostaining for osteopontin and osteocalcin, i.e., markers of osteogenic cell differentiation (Grausová *et al*., 2008b). All these results were corroborated by studies performed on fullerene $C_{60}$ layers deposited onto polystyrene culture dishes, which supported adhesion, viability and growth of breast epithelial cells (Levi *et al*., 2006), and also by studies on fullerenes grafted onto polyurethane, which enhanced adhesion and activation of platelets (Lin and Wu, 1999).

### 7.1.2. Micropatterned fullerene $C_{60}$ films

In the second set of studies, we investigated the adhesion, growth, viability and maturation of human MG 63 osteoblast-like cells on micropatterned layers prepared by deposition of fullerenes on microscopic glass coverslips through contact metallic masks. In this case, the fullerenes formed bulge-like prominences of various heights (from 128 ± 8 nm to 1043 ± 57 nm) on the material surface. All tested micropatterned fullerene layers proved to be suitable substrates for the adhesion and growth of MG 63 cells. On layers with prominences between 128 ± 8 nm and 326 ± 5 nm in height, the cells grew with similar doubling times and population densities as on the tissue culture polystyrene and glass coverslips, which were used as control samples. On these layers, the cells were distributed homogeneously, thus this surface morphology did not induce a specific cell distribution. However, on the fullerene surface with the highest prominences of 1043 ± 57 nm, the cells grew preferentially in the grooves among the prominences. Although these grooves occupied only approximately 41 % of the surface, they contained from 80 % to 98 % of the cells, and the cell population density in the grooves was about 5 to 57 times higher than on the bulges. In other words, the cells were not able to "climb up" fullerene prominences higher than 1 μm. This may be due to a combination and synergetic action of several factors less appropriate for cell adhesion, such as hydrophobicity, the ball-like shape of the fullerenes, and their diffusion out of the prominences (Grausová *et al*., 2008a). From these studies it follows that microstructured fullerene layers with relatively high fullerene prominences could be used for applications where regionally-selective adhesion and directed growth of cells is required, e. g. in tissue engineering, in cell microarrays for genomics and proteomics, or for constructing biosensors. On all surfaces micropatterned with fullerenes $C_{60}$, the LIVE/DEAD viability/cytotoxicity kit staining showed that the cell viability was high on all tested fullerene substrates. Similarly as in continuous fullerene $C_{60}$ films, the cells on all tested micropatterned surfaces were able to form $\beta_1$ integrin- and talin-containing focal adhesion plaques, a β-actin cytoskeleton, and to produce osteocalcin and osteopontin, i.e., markers of differentiation of osteogenic cell types (Bačáková *et al*., 2008; Grausová *et al*., 2008 a).

The biocompatibility and bioactivity of fullerene layers can be further enhanced by functionalization with various chemical groups. For example, a bisphosphonate fullerene $C_{60}(OH)_{16}$AMBP has been found to bind hydroxyapatite, and thus it might be used for supporting bone mineralization (González *et al*., 2002).

### 7.1.3. Hybrid $C_{60}$/Ti films with a continuous and micropatterned morphology

For applications in bone tissue engineering, fullerenes can be used in combination with titanium. As mentioned above (for more details see chapter 2.2.), titanium is well known to be appropriate for hard tissue surgery. In our third set of experiments on fullerenes, we investigated the adhesion and growth of MG 63 osteoblast-like cells on continuous and micropatterned $C_{60}$ and binary $C_{60}$/Ti films. Some micropatterned $C_{60}$/Ti films were irradiated with $Au^+$ ions (energy 1.8 MeV, fluence 2 x $10^{14}$ $cm^{-2}$), which led to the conversion of some fullerene molecules into amorphous carbon.

On day 1 after seeding, the highest cell numbers were observed on continuous $C_{60}$/Ti and pure $C_{60}$ films irradiated with $Au^+$ ions. This may be explained by an increased content of oxygen and thus increased wettability, which enhances cell adhesion (for more details see chapter 3.2.). Fullerenes undergo photooxidation when exposed to light, and titanium is easily oxidized in ambient atmospheres (Vandrovcová *et al*., 2008).

One and three days after seeding, the cells on all tested $C_{60}$ and binary $C_{60}$/Ti films with a continuous or micropatterned morphology reached numbers which were usually similar to, or even higher than, the values on the control glass coverslips and standard cell culture polystyrene dishes. On both micropatterned $C_{60}$ and hybrid $C_{60}$/Ti layers, the cells adhered and grew preferentially in the grooves among the prominences (~ 57 to 90 % of cells). Interestingly, on day 3 after seeding, this preferential colonization of the grooves disappeared in the ion-irradiated layers, which could be explained by the conversion of $C_{60}$ into amorphous carbon and increased hydrophilia of the layers. Thus, similarly as in our earlier experiments described above, all tested $C_{60}$ and $C_{60}$/Ti films gave good support to the adhesion and growth of bone-derived MG 63 cells. In addition, microstructured $C_{60}$ and $C_{60}$/Ti films, containing prominences and grooves on their surface, could be applied for regionally-selective cell adhesion and directed cell growth. For this purpose, we also prepared some surfaces that were not fullerene-based, namely surfaces with hydrophilic strips created by plasma polymerization of acrylic acid, and with hydrophobic strips created from 1,7-octadiene. The hydrophilic microdomains supported preferential adhesion and maturation in mesenchymal stem cells derived from the bone marrow of pigs, and also in various other cell types, such as rat vascular smooth muscle cells, bovine pulmonary artery endothelial cells and human skeletal myoblasts (Filová *et al*., 2008).

Author's publications relevant to the part of the thesis dealing with $C_{60}$ and $C_{60}$/Ti films:

Bačáková L., Grausová L., Vacík J., Fraczek A., Blazewicz S., Kromka A., Vaněček M., Švorčík V. Improved adhesion and growth of human osteoblast-like MG 63 cells on biomaterials modified with carbon nanoparticles. **Diamond Relat. Mater.** 2007; 16: 2133-2140. Impact factor 2.0

Bačáková L., Grausová L., Vandrovcová M., Vacík J., Frazcek A., Blazewicz S., Kromka A., Vaněček M., Nesládek M., Švorčík V., Kopeček M.: Carbon nanoparticles as substrates for cell adhesion and growth. **In: Nanoparticles: New Research.** Frank Columbus, Ed., Nova Science Publishers, Inc., Hauppauge, New York, 2008, accepted, in press

Filová E., Bullett N.A., Bačáková L., Grausová L., 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. **Physiol. Res**., accepted, in press. Impact factor 1.5

Grausová L., Vacík J., Bílková P., Vorlíček V., Švorčík V., Soukup D., Bačáková M., Lisá V., and Bačáková L.: Regionally-selective adhesion and growth of human osteoblast-like MG 63 cells on micropatterned fullerene $C_{60}$ layers. **J. Optoelectron. Adv. Mater.** 2008a; 10: 2071-2076. Impact factor 1.1

Grausová L., Vacík J., Vorlíček V., Švorčík V., Slepička P., Bílková P., Vandrovcová M., Lisá V. and Bačáková L. Fullerene $C_{60}$ films of continuous and micropatterned morphology as substrates for adhesion and growth of bone cells. **Diamond Relat. Mater**. 2008b; accepted, in press. Impact factor 2.0

Vandrovcová M., Vacík J., Švorčík V., Slepička P., Kasálková N., Vorlíček V., Lavrentiev V., Voseček V., Grausová L., Lisá V. and Bačáková L. Fullerene $C_{60}$ and hybrid $C_{60}$/Ti films as substrates for adhesion and growth of bone cells. **Phys. Stat. Sol. (a)** 2008; 205: 2252-2261. Impact factor 1.2

### 7.2. Interaction of cells with carbon nanotube-polymer composites

Carbon nanotubes are a carbon allotrope which has become very attractive for potential applications in tissue regeneration, and have been much more deeply investigated in this field than fullerenes. They have been widely studied in their modified form and in interaction with various cell types. Our study focused on the adhesion, growth and differentiation of osteoblast-like MG 63 cells grown on composites of carbon nanotubes with a non-degradable terpolymer. In these materials, single-wall carbon nanohorns (SWNH) or high crystalline electric arc multi-wall nanotubes (MWNT-A) in concentrations from 2 wt% to 8 wt% were mixed with a terpolymer of polytetrafluorethylene, polyvinyldifluoride and polypropylene (PTFE/PVDF/PP).

We have found, that on the terpolymer-nanotube composites, the MG 63 cells were well spread, polygonally shaped, and contained a well-developed beta-actin cytoskeleton. In contrast, most of the cells cultivated on the pure terpolymer were small, rounded and clustered into aggregates, which indicated low cell viability or even apoptotic death. The improved adhesion and growth of MG 63 cells on carbon nanotube-terpolymer composites may be explained by the supportive effect of nanorough surfaces mimicking the architecture of the natural extracellular matrix (for more details see chapter 3.2.). These terpolymer-nanotube composites remained similar in hydrophobicity to pristine unmodified terpolymer (water drop contact angle about $100^{o}$), but due to the surface nanostructure, the cell adhesion-mediating ECM proteins were probably adsorbed in a geometrical conformation appropriate for their accessibility with cell adhesion receptors.

Accordingly, the MG 63 cells cultivated on the composite with 4 wt% of SWNH contained higher concentrations of vinculin and talin (i.e., integrin-associated proteins and important components of focal adhesion plaques) in comparison with the values in cells grown on a polystyrene culture dish, and also those grown on the pure terpolymer. The amount of osteocalcin, i.e., a marker of osteoblast differentiation, was similar in cells cultivated on all materials except the composites containing 4 wt% and 6wt% of MWNT-A, where the concentration of osteocalcin was significantly lower in comparison with the concentration in cells on the pure terpolymer and on a polystyrene culture dish. The lower concentration of osteocalcin in cells on the composite with MWNT-A may be explained by the higher rate of proliferation, which probably delayed the differentiation process (Bačáková *et al*., 2007; for a review, see Bačáková *et al*., 2008).

The MG 63 cells grown on the pure terpolymer and all carbon nanotube-terpolymer composites showed no immune activation. One of the important markers of cell immune

activation, i.e., ICAM-1, a receptor for inflammatory cells, was not increased in cells on SWNH and MWNT-A-terpolymer composites, or on the pure terpolymer (Bačáková *et al.*, 2007).

Our findings are in agreement with the results of Zanello and co-workers, who also demonstrated that carbon nanotubes (in the form of a scaffold) support the adhesion and proliferation of rat osteosarcoma-derived ROS17/2.8 cells (Zanello *et al*., 2006).

Carbon nanotubes could be also prepared in the form of composites with degradable polymers such as chitosan. Abarrategi and co-workers investigated mouse myoblast C2C12 cells on MWNTs-chitosan composites with adsorbed recombinant human bone morphogenetic protein-2 (rhBMP-2). This composite gave good support to cell adhesion, spreading, proliferation and differentiation of C2C12 cells toward an osteoblastic phenotype. The material was then implanted into a subcutaneous pocket made in the mouse back muscle tissue. After 3 weeks, there was bone tissue regeneration and there was no inflammatory reaction. This was consistent with our *in vitro* studies, which showed that carbon nanotube-polymer composites supported osteoblast growth and were non-immunogenic (Abarrategi *et al*., 2008). Inspired with these encouraging results, we attempted to construct similar composites of carbon nanotubes with degradable polymers, i.e. a polylactide-co-glycolide (PLGA) copolymer (Pamula *et al*., 2008). Composites of carbon nanotubes and polylactic acid were successfully used for cultivating primary rat calvaria osteoblasts and for stimulating them electrically (Supronowicz *et al*., 2002). Therefore, we expected that in our three-dimensional porous PLGA scaffolds, the carbon nanotubes could decorate the pore walls, and the nanostructure of these walls would simulate the ingrowth and maturation of osteogenic cells. At the same time, the nanotubes would reinforce the polymerc material, which is less appropriate for bone tissue engineering in its non-modified form (Pamula *et al*., 2008).

The cell adhesion, proliferation and differentiation on carbon nanotube-based materials can be significantly improved by binding specific functional groups or biomolecules to the carbon nanotube surface, e.g. oxygen-containing groups (Balani *et al*., 2007), aminoacids (Georgakilas *et al*., 2002), proteins (Chakravarty *et al*., 2008), or growth factors, such as bone morphogenetic protein (Abarrategi *et al*., 2008).

Despite all these encouraging findings, the use of both fullerenes and nanotubes in tissue regeneration is compromised by their potential cytotoxic and immunogenic effects. At the present time, nanodiamond films and powders seem to be the most promising carbon nanotube and fullerene material. They have shown excellent biocompatibility and no cytotoxicity and immunogenicity.

Author's publications relevant to the part of the thesis dealing with carbon nanotube-polymer composites:

Bačáková L., Grausová L., Vacík J., Fraczek A., Blazewicz S., Kromka A., Vaněček M., Švorčík V. Improved adhesion and growth of human osteoblast-like MG 63 cells on biomaterials modified with carbon nanoparticles. **Diamond Relat. Mater.** 2007; 16: 2133-2140. Impact factor 2.0

Bačáková L., Grausová L., Vandrovcová M., Vacík J., Frazcek A., Blazewicz S., Kromka A., Vaněček M., Nesládek M., Švorčík V., Kopeček M.: Carbon nanoparticles as substrates for cell adhesion and growth. **In: Nanoparticles: New Research.** Frank Columbus, Ed., Nova Science Publishers, Inc., Hauppauge, New York, 2008, accepted, in press

Pamula E., Bačáková L., Filová E., Buczynska J., Dobrzynski P., Nosková L., Grausová L. The influence of pore size on colonization of poly(L-lactide-glycolide) scaffolds with human osteoblast-like MG 63 cells in vitro. **J. Mater. Sci. Mater. Med.** 2008; 19: 425-435. Impact factor 1.6

### 7.3. Interaction of cells with nanocrystalline diamond films

Nanocrystalline diamond and diamond-like carbon have already been used for coating the heads and cups of artificial joint replacements, e.g. metallic and polymeric prostheses of hip or temporomandibular joints (Lappalainen *et al*., 2003; Papo *et al*., 2004). In these cases, diamond films were applied on surfaces which require mechanical stability but do not enter into direct contact with cells. However, nanostructured diamond layers could also be used for coating the bone-anchoring stems of joint prostheses and other bone implants in order to improve their bioactivity and thus their integration with the surrounding bone tissue. Therefore, we investigated the growth of osteoblast-like MG 63 cells, as well as vascular endothelial cells, i.e. another important component of the bone tissue, on nanocrystalline diamond (NCD) layers with two different surface topographies. NCD films were grown on silicon substrates by a microwave plasma enhanced chemical vapor deposition (PECVD) method.

The first type of NCD layer was nanostructured, having root mean square roughness (rms) 8.2 nm. The second NCD layer was characterized by a hierarchically- organized micro- and nanostructure (rms 301 nm and 7.6 nm), i.e., by micro-sized irregularities bearing

nanoroughness of their surface. The reason for the use of these layers was that a hierarchical organization on two or multiple length scales is the main architectural principle of building natural tissues, including bone. In addition, it is known from several studies that not only the nanoroughness of the material surface but also its microstructure is important for its colonization with osteoblasts, and for regeneration of the adjacent bone tissue. Surface roughness on the submicron or micrometer scale has several times been shown to enhance the strength of osteoblast adhesion, spreading and differentiation (Kim *et al*., 2005; Sammons *et al*., 2005; Zhao *et al*., 2005, 2006), including the deposition of mineralized bone extracellular matrix (Boyan *et al*., 2002) and acquisition of osteoblast phenotype in mesenchymal and osteoprogenitor cells (Lohmann *et al*., 2000; Schneider *et al*., 2004).

Our results have shown that MG 63 cells were viable, well spread and homogeneously distributed on both NCD layers. The cell number and viability on day 1 after seeding were similar to the corresponding parameters in cells grown on the control polystyrene culture dishes. The cell population on both NCD layers increased rapidly, and on day 3 after seeding it reached significantly higher values than on the control polystyrene. In addition, the cell viability on both NCD layers reached more than 99%. On day 5 after seeding, both NCD layers were fully covered with cells with almost 100% viability. In contrast, the bare silicon substrates (i.e., without NCD coatings), were revealed to be cytotoxic, and the cell number and viability on these substrates has a time-dependent decreasing tendency. The cells cultivated on silicon substrates were round, and were often clustered into aggregates, indicating cell dysfunction and death. On day 5, the number of cells on the silicon substrates was extremely low and the cell viability was almost 0% (Grausová *et al*., 2008c, d).

Similar results were obtained with silicon substrates implanted into rabbit eyes, where the action of these materials was highly corrosive. When these silicon substrates were covered with NCD layers, their action was highly biocompatible, similarly as in our study (Xiao *et al*., 2006). The termination of our tested NCD layers with oxygen, resulting in relatively high hydrophilicity, probably also played a significant role in good cell colonization. In our previous studies dealing with the interaction of MG 63 cells, vascular endothelial and smooth muscle cells with ion-implanted or UV-irradiated polymers or carbon-titanium coated CFRC, the presence of various oxygen-containing chemical functional groups on the surface increased the cell adhesion, growth and expression of cell-type specific differentiation markers (Bačáková *et al*., 2000a,b; Bačáková *et al*., 2001a,b; Heitz *et al*., 2003).

The high viability and metabolic activity of cells on NCD layers was also demonstrated using the MTT test, which detects the function of mitochondrial enzymes. This

test indicated that the mitochondria in cells on both NCD surfaces were intact and well functioning. In addition, the activity of the mitochondrial enzymes in cells cultivated on both types of NCD films was significantly higher than in cells grown on the control polystyrene. Our results obtained from LIVE/DEAD and MTT tests are compatible with the results of several previous studies. Amaral and co-workers, who also have tested the biocompatibility of nanodiamond particles on MG 63 cells, demonstrated that these cells in cultures on NCD films were well-attached and spread, displaying their typical morphology. These authors also reported that the activity of mitochondrial enzymes was higher in cells on NCD coatings than on cells on standard polystyrene culture plates. In addition, NCD stimulated the activity of alkaline phosphatase and subsequent matrix mineralization (Amaral *et al*., 2008). Similarly, Bajaj *et al*. reported that NCD surfaces are preferred by osteoblast-like MC3T3 cells in comparison with cervical carcinoma HeLa cells or pheochromocytoma PC12 cells (Bajaj *et al*., 2007). The good biocompatibility of nanocrystalline diamond has also been studied using neuroblastoma cells and macrophages. Schrand *et al*. demonstrated that no morphological changes or signs of cell death were found in neuroblastoma cells and macrophages (Schrand *et al*., 2007).

Our studies also showed that on day 3 after seeding, talin and beta-actin were organized into well-developed focal adhesion plaques and cytoskeletal fibres, respectively, in MG 63 cells cultivated on both NCD films and the control polystyrene. The cell spreading area of MG 63 cells grown on nano-structured NCD (2 744 ± 116 $\mu m^2$) was similar to the spreading area of cells grown on the microscopic glass coverslips (2 742 ± 133 $\mu m^2$) used as a reference material, but the size of the cells on the hierarchically micro- and nanostructured NCD substrates was significantly larger (3 733 ± 179 $\mu m^2$; Grausová *et al*., 2008c, d). The concentration of talin, i.e. a focal adhesion protein taking an important part in cell adhesion and spreading, was significantly higher in cells on hierarchically-organized submicron-nano-NCD (by 36.0 ± 6.2% compared to the value on the polystyrene dishes). In addition, the concentration of vinculin increased on both nano-NCD and submicron-nano-NCD (by 19.8 ± 3.4 % and 26.0 ± 6.1%, respectively; Grausová *et al.*, 2008e). These results agree with a study reporting a supportive effect of micro-structured dental titanium implants on the spreading of rat osteoblasts in primary cultures (Sammons *et al*., 2005) and the development of polygonal shape in osteoblasts (Sader *et al*., 2005).

In contrast, in our previous study performed on carbon fiber-reinforced carbon composites (CFRC), the size of the spreading area of MG 63 cells and rat vascular smooth muscle cells correlated inversely with the surface microroughness (rms from 3.25 ± 0.35 μm

to 0.35 ± 0.09 μm), and on the surfaces with the highest roughness, the cells were mostly spindle-shaped. An elongated spindle-shaped morphology in osteoblats has often been considered as a more differentiated phenotype (Kim *et al*., 2005; Zhao *et al*., 2006), but in our earlier studies, elongated MG 63 cells grown on relatively rough surfaces contained a lower concentration of osteocalcin (Starý *et al.,* 2003a).

Our studies also showed that NCD films seem to be suitable for adhesion and growth of vascular endothelial cells. The NCD films were homogeneously covered with CPAE cells with high viability, as in the case of MG 63 cells. However, the CPAE cells were more sensitive to surface roughness than the MG 63 cells, and their adhesion and growth was better on nanostructured NCD films than on hierarchically micro- and nanostructured NCD films. This was probably because the bovine arterial endothelial cells were larger than human bone-derived cells, and thus required more space for spreading, which was limited by the relatively big and sharp irregularities (Grausová *et al.,* 2008a).

Interestingly, the CPAE cells were less sensitive to the cytotoxic action of silicon substrates. In contrast with MG 63 cells, the viability of CPAE cells on both silicon substrates increased significantly with time of cultivation, reaching almost 100% on day 5 after seeding. From previous studies it was reported that the cytotoxicity of silica nanoparticles is dependent on the metabolic activity of a given cell type. For example, fibroblasts with relatively long doubling times were more susceptible to injury induced by silica exposure than tumor cells with short doubling times (Chang *et al*., 2007).

According to our own and other studies, nanodiamonds in the form of films seem to be very promising in bioartificial implant construction applications. Hard, mechanically resistant and biocompatible films of nanocrystalline diamond, developed in this study, could be used in practical applications for coating the bone-anchoring stems of joint prostheses in order to improve their integration with the bone tissue. Nanodiamonds could also be very useful for constructing three-dimensional porous implants. Like carbon nanotubes, nanodiamond could also be admixed into a polymeric matrix, and after formation of the scaffolds, they could decorate the pore walls (i.e., form nanoscale prominences on the pore walls) and improve the adhesion, growth and differentiation of bone cells (Bačáková *et al*., 2007, 2008; Grausová *et al*., 2008c, d, e). In addition, we used nanostructured NCD layers for constructing micropatterned surfaces for guided cell adhesion and growth. These surfaces were patterned with strips of hydrophilic NCD terminated with oxygen and hydrophobic H-terminated NCD. Human SaOs-1 osteoblasts adhered and grew preferentially on the hydrophilic O-terminated microdomains (Rezek *et al.*, 2008).

Author's publications relevant to the part of the thesis dealing with nanocrystalline diamond films:

Bačáková L., Grausová L., Vacík J., Fraczek A., Blazewicz S., Kromka A., Vaněček M., Švorčík V. Improved adhesion and growth of human osteoblast-like MG 63 cells on biomaterials modified with carbon nanoparticles. **Diamond Relat. Mater.** 2007; 16: 2133-2140. Impact factor 2.0

Bačáková L., Grausová L., Vandrovcová M., Vacík J., Frazcek A., Blazewicz S., Kromka A., Vaněček M., Nesládek M., Švorčík V., Kopeček M.: Carbon nanoparticles as substrates for cell adhesion and growth. **In: Nanoparticles: New Research.** Frank Columbus, Ed., Nova Science Publishers, Inc., Hauppauge, New York, 2008, accepted, in press

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Rezek B., Michalíková L., Kromka A., Kalbáčová M., Kmoch S., Grausová L., Bačáková L., Vaněček M., Kočka J.: Způsob přípravy uspořádaných buněčných struktur (Mode of preparation of organized cell structures). **Patent Application,** Industrial Property Office, (Czech Patent and Trademark Office), Prague, Czech Rep.; registration number PV 2008-355, 2008.

## 8. CONCLUSIONS

All carbon nanoparticle-containing materials inverstigaetd in this study, i.e. fullerene C60 layers, carbon nanotube-polymer composites and nanocrystalline diamond films, provided good support for the adhesion, growth, viability, metabolical activity and maturation of bone-derived cells. At the same time, these materials did not promote significant immune activation of these cells. More detailed concluding remarks:

- Human osteoblast-like MG 63 cells cultivated on CFRC composites covered with a continuous fullerene $C_{60}$ layer adhered in lower numbers but with a large cell spreading area. These cells formed a well-developed network of beta-actin filaments and vinculin-containing focal adhesion plaques.
- Fullerenes $C_{60}$ deposited on microscopic glass coverslips as layers micropatterned with grooves and prominences from 128 ± 8 nm to 326 ± 5 nm in height supported the adhesion, growth and viability of MG 63 cells, which were distributed homogeneously on these layers.
- Fullerene $C_{60}$ layers with prominences 1043 ± 57 nm in height promoted regionally-selective adhesion and growth of MG 63 cells in the grooves located among the prominences.
- Hybrid fullerene $C_{60}$/Ti films supported adhesion, proliferation and viability of MG 63 cells. When micropatterned with grooves and bulge-like prominences, they induced preferential colonization with MG 63 cells in the grooves, similarly as the micropatterned layers constructed from pure $C_{60}$.
- Composites of single-walled or multiwalled carbon nanotubes with a terpolymer of polytetrafluoroethylene, polypropylene and polyvinyldifluoride markedly improved the adhesion, spreading, assembly of a beta-actin cytoskeleton, and viability of MG 63 cells in comparison with pure terpolymer.
- The highest concentration of vinculin and talin (i.e., markers of cell adhesion) was detected in cells cultivated on terpolymer with single-walled carbon nanohorns (SWNH).
- There was a similar concentration of osteocalcin (a marker of osteogenic differentiation) in cells on SWNH-modified terpolymer, on pure terpolymer and on the polystyrene culture dish.

- In cells on terpolymer with multi-wall carbon nanotubes (MWNT-A), the concentration of osteocalcin was significantly lower than the concentration in cells on pure terpolymer and on the polystyrene culture dish (probably due to high proliferation activity of the cells)
- The concentration of ICAM-1, a marker of cell immune activation, was not increased in cells on the two carbon nanotube-terpolymer composites and on pure terpolymer.
- Nanocrystalline diamond (NCD) films with a surface nanostructure or a hierarchically-organized micro- and nanostructure gave good support for the adhesion and growth of osteogenic cells.
- The viability of ostoblast-like MG 63 cells on the two NCD films was very high (almost 100%, on day 5 after seeding). On the other hand, the viability of these cells on bare silicon substrates without NCD layers had a time-dependent decreasing tendency.
- The activity of mitochondrial enzymes in MG 63 cells cultivated on the two NCD layers was significantly higher than the activity of these enzymes in cells cultured on a control polystyrene culture dish.
- The adhesion and growth of osteogenic MG 63 cells was supported by the hierarchically-organized microroughness and nanoroughness of the material, whereas colonization with vascular endothelial CPAE cells was better on purely nanostructured NCD surfaces.
- In contrast to MG 63 cells, the viability of CPAE cells on silicon substrates was relatively high and comparable to that on a polystyrene culture dish. On day 5, the viability of CPAE cells on silicon substrates was almost 100%.
- The MG 63 cells on NCD films contained a higher concentration of focal adhesion proteins talin and vinculin. The concentrations of osteocalcin and ICAM-1 were similar to those on the control polystyrene dishes.

## 9. SUMMARY

Recently, nanotechnology in tissue engineering has become a very important field of study. Many different materials of nanoscale roughness have been studied for their potential use in the regeneration of various tissues. The reason is that nanostructured materials imitate the architecture of natural extracellular matrix and thus support cell adhesion, growth and differentiation. Our investigation focused on the influence of fullerene layers, carbon nanotube-terpolymer composites and nanocrystalline diamond layers on the adhesion, growth and differentiation of human osteoblast-like MG 63 cells and bovine vascular endothelial CPAE cells. Each of these materials supported colonization with cells. On continuous fullerene $C_{60}$ layers, deposited on composites with the carbon matrix reinforced with carbon fibres, the cell population density was lower than on non-coated composites, but MG 63 cells were well-spread with well-developed focal adhesion plaques and a beta-actin cytoskeleton. On continous fullerene $C_{60}$ layers deposited on microscopic glass coverslips, the adhesion, growth, viability and osteogenic maturation of MG 63 cells was similar as on the control uncoated glass and standard polystyrene cell culture dishes. Similar behavior of MG 63 cells was observed on hybrid $C_{60}$/Ti films. Microstructured fullerene $C_{60}$ and hybrid $C_{60}$/Ti layers, containing bulge-like prominences and grooves, promoted preferential adhesion and growth of MG 63 cells in the grooves. On composites of carbon nanotubes with a terpolymer of polytetrafluoroethylene, polypropylene and polyvinyldifluoride, the adhesion, spreading, formation of foca adhesion plaques and a beta-actin cytoskeleton, viability and cell growth were markedly improved in comparison with pure terpolymer. At the same time, these cells did not show significant immunological activation, measured by the concentration of ICAM-1 immunoglobulin molecule. Nanostructured and especially hierarchically micro- and nanostructured nanocrystalline diamond (NCD) films markedly supported the adhesion, growth, viability and metabolic activity of MG 63 cells. These cells showed a higher concentration of talin and vinculin (measured per mg of protein), higher activity of mitochondrial enzymes, and very high viability. In contrast, the viability of MG 63 cells on uncoated silicon substrates was very low and had a time-dependent decreasing tendency. On the other hand, the viability of CPAE cells on silicon substrates was high, and increased with time to almost 100%. However, the colonization with endothelial cells was better on purely nanostructured NCD films than on hierarchically micro- and nanostructured NCD films, because the microscale surface irregularities probably hampered cell spreading.

Nevertheless, taken together, all carbon nanoparticle-based materials studied here provided good support for colonization with bone-forming cells, and thus they could be used for constructing bone implants, e.g. as bioactive material coatings or as components of polymeric scaffolds for bone tissue engineering. In addition, fullerenes could be utilized for creating micropatterned surfaces if regionally-selective adhesion and directed cell growth are needed.

## 10. ABBREVIATIONS

| | |
|---|---|
| A431 | human epitheloid carcinoma cells |
| ALP | alkaline phosphatase |
| Ag | antigene |
| B | lymphoblast cell line |
| BASD | bead-assisted sonic disintegration |
| bcl-2 | antiapoptotic protein |
| BMP-2 | bone morphogenetic protein 2 |
| BSA | bovine serum albumin |
| C2C12 | mouse myoblast cell line |
| CFRC | carbon fiber reinforced carbon |
| CNS | central nervous system |
| CNTs | carbon nanotubes |
| CPAE | calf pulmonary artery endothelial cells |
| CVD | chemical vapour deposition |
| DMEM | Dulbecc's modified Eagle medium |
| DNA | deoxyribonucleotic acid |
| dRib | 2-deoxy-D-ribose |
| DMSO | dimethylsulfoxide |
| ECM | extracellular matrix |
| GCT | glutamate-cysteine ligase |
| GST | gluthatione-S-transferase |
| $GH_3$ | rat pituitary tumor cells |
| HA | hyaluronic acid |
| HAP | hydroxyapatite |
| HeLa | human cervix uteri tumor-derived cells |
| HEp-2 | human epithelial-like tumor cell line |
| HEp-3B | human hepatoma cell line |
| hFOB | human fetal osteoblastic cell line |
| HIV | human immunodeficiency virus |
| hMSC | human mesenchymal stem cells |
| 4-HNE | 4-hydroxynonenal |

| | |
|---|---|
| HUVECs | human umbilical vein endothelial cells |
| ICAM-1 | intercellular adhesion molecule-1 |
| IgE | immunoglobuline E |
| IL-1 | interleukin 1 |
| KGAGDV | Lys-Gly-Ala-Gly-Asp-Val |
| KRSR | Lys-Arg-Ser-Arg |
| LB | lymphoblast cell line |
| MALDI-TOF | Matrix Assisted Laser Desorption/Ionization Time-of-Flight |
| MAP | mussel adhesive protein |
| MC | mast cells |
| MC3T3 | mouse calvarial cell line |
| MC3T3-E1 | mouse osteoblast cell line |
| MG 63 | human osteosarcoma cells |
| MSTO-211H | human mesothelioma cell line |
| MTT | 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide |
| MWNT-A | arc multi-wall nanotubes |
| MWNTs | multi- walled nanotubes |
| MW PECVD | microwave plasma-enhanced chemical vapour deposition |
| NCD | nanocrystalline diamond |
| NMDA | N-methyl-D-aspartate |
| O-NCD | oxygen-terminated nanocrystalline diamond |
| PBMCs | human peripheral blood mononuclear cells |
| PC12 | rat pheochromocytoma cells |
| PCL | polycaprolactone |
| PCU | polycarbonate urethane |
| PDS | polydioxanone |
| PECVD | plasma enhanced chemical vapor deposition |
| PEG | polyethylene glycol |
| PET | polyethylene terephthalate |
| PGA | poly(glycolic acid) |
| PHB | poly(hydroxybutyrate) |

| | |
|---|---|
| PHEMA | poly(hydroxyethyl methacrylate) |
| PHV | polyhydroxyvalerate |
| PLA | poly(lactic acid) |
| PMN | polymorphonuclear leucocytes |
| PP | polypropylene |
| PTFE | polytetrafluorethylene |
| PU | polyurethane |
| PVDF | polyvinyldifluoride |
| RASMCs | rat aortic smooth muscle cells |
| REDV | Arg-Leu-Asp-Val |
| RGDV | Arg-Gly-Asp |
| rhBMP-2 | recombinant human bone morphogenetic protein-2 |
| ROS | reactive oxygen species |
| ROS17/2.8 | rat osteosarcoma-derived cell line |
| SaOs-2 | human osteosarcoma cells |
| SWNHs | single-walled nanohorns |
| SWNTs | single-walled nanotubes |
| 3T3 | mouse fibroblast cell line |
| TCP | tricalcium phosphate |
| TGF-β | tumor growth factor- β |
| TNF-α | tumor necrotic factor α |
| UNCD | ultra nanocrystalline diamond (UNCD) |
| UV | ultra violet irradiation |
| UVB | ultra violet irradiation type B |
| VAPG | Val-Ala-Pro-Lys |

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## 13. SCHOLARSHIPS

**3.4.-16.4.2005**: AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Krakow, Poland (Prof. S. Blazewicz, Ewa Stodolak, PhD.)

**2.4.-15.4.2006**: AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials, Cracow, Poland (Prof. M. Blazewicz, Aneta Fraczek, PhD.)

**3.7.-15.7.2006**: International School **NanoSciencesTech:** SUMMER SCHOOL ON NANOTUBES. Cargése, Corsica, France.