

Osteogenic cells on bio-inspired materials for bone tissue engineering

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Summary

This article reviews the development of artificial bone substitutes from their older single-phase forms to novel multi-phase composites, mimicking the composition and architecture of natural bone tissue. The new generation of bone implants should be bioactive, i.e. they should induce the desired cellular responses, leading to integration of the material into the natural tissue and stimulating self-healing processes. Therefore, the first part of the review explains the common principles of the cell-material interaction and summarizes the strategies how to improve the biocompatibility and bioactivity of the materials by modifying the physico-chemical properties of the material surface, such as surface chemistry, wettability, electrical charge, rigidity, micro-roughness and especially nano-roughness. The latter has been shown to stimulate preferentially the growth of osteoblasts in comparison with other competitive cell types, such as fibroblasts, which could prevent fibrous tissue formation upon implantation. The second more specialized part of the review deals with materials suitable for bone contact and substitution, particularly novel polymer-based composites reinforced with fibres or inorganic particles and containing bioactive components, such as crystals of hydroxyapatite or other calcium phosphates, synthetic ligands for cell adhesion receptors or growth factors. Moreover, if they are degradable, they can be gradually replaced with a regenerating tissue.

Key Words: multi-phase composites, nanoroughness, osteoblasts, bone implants, bioartificial bone

1. Introduction

Biomaterial science and tissue engineering have developed as new and independent interdisciplinary scientific fields in response to the rising demand for replacements of damaged tissue in the growing and aging population.

In the case of bone tissue loss as a result of bone diseases or traumatic damage, several strategies are applied. Currently, the most widely used are autologous transplantations, using bone grafts from the same patient. However, this has several serious disadvantages, for example the patient is subjected to an additional surgical procedure, as well as prolonged rehabilitation and healing time, increased pain and risk of infection (Mata *et al.* 2002); but most importantly the amount of available material is limited as the patient's bone tissue is basically being damaged at another site. Allogenic or xenogenic grafts or materials are in general unsuitable, because of the possible immune response and subsequent rejection, as well as the possibility of disease transmission. Therefore, great attention is being paid to the development of artificial materials that could possibly replace and substitute the damaged or lost bone tissue.

Apart from the mechanical properties that are imposed on these implanted artificial materials, or biomaterials, the main requirement is their biocompatibility. This means they should be accepted by the surrounding tissues and by the body as a whole. In other words, the materials should be non-toxic, non-immunogenic and non-carcinogenic (Park and Bronzino 2003).

During the history of biomaterial engineering, a range of approaches has been applied. The earliest so-called first-generation materials were designed as bioinert. The main objective was to create a material that would match the mechanical properties of the replaced tissue, and would not allow protein adsorption and cell adhesion, in order to reduce the possible immune response and rejection (Hench and Polak 2002). However, modern advanced materials, sometimes referred to as second-generation biomaterials, are specifically designed to be "bioactive". This means they should

elicit specific desired cellular responses, like cell adhesion, proliferation and differentiation into a specific cell type, e.g., bone cells that will form a new bone tissue and thus integrate the implant strongly into the surrounding natural tissue. The reaction of the cells should be controllable by the physical and chemical properties of the material surface (Hench and Polak 2002, for a review see Bačáková *et al.* 2004a).

One of the most advanced strategies in recent research in tissue engineering is the construction of 3-dimensional porous scaffolds made of resorbable materials, especially polymers. These 3-dimensional porous scaffolds should be seeded with the patient's own cells or even stem cells, e.g., those derived from the bone marrow taken under biopsy from the iliac crest, and then expanded in cell culture conditions. Upon implantation into the body, these hybrid cell-material constructs should gradually replace the missing bone by completely newly formed tissue. The polymeric scaffolds that provide the cells with the necessary support during this self-healing process should gradually degrade, as they will be continuously replaced by new bone and will eventually disappear completely (Bačáková *et al.* 2004a). Some authors refer to these materials as third-generation biomaterials, because they will stimulate the specific response of cells at a molecular level, and activate specific gene expression that regulates regeneration and the self-healing process (Hench and Polak 2002). However, in the case of polymer-based bone constructs, their potential use is still very limited due to their insufficient mechanical properties as load-bearing implants (Kim *et al.* 2006, Rezwan *et al.* 2006, Boccaccini and Blaker 2005). These materials need further improvements, e.g. strong mechanically resistant reinforcement with fibrous or particulate component and loading with bioactive molecules which would accelerate the formation of regenerated, mineralized and fully functional bone tissue.

In order to achieve all the desired and regulated cellular responses, the mechanism of cell interaction with the surface of an artificial material must first be well understood. Therefore, the first section of this article reviews the molecular mechanisms of interaction between cells and

artificial materials, which is strongly dependent on physical and chemical properties of the material surface. The second part of this review then follows the development of the bone tissue substitutes from their older and usually single-phase forms to advanced bioactive multiphase composites inspired by the composition and architecture of the natural bone tissue.

2. Common principles of the cell-material interaction

2.1. Protein adsorption and physicochemical properties of the material surface

Immediately after the biomaterial is implanted into an organism or comes into contact with cell culture environments, protein adsorption to its surface occurs. This happens within seconds, long before the first cells reach the surface. Consequently, cells almost never come into direct contact with the material surface; they rather interact with the layer of adsorbed proteins. This layer mediates the cell adhesion, and also provides signals to the cell through the cell adhesion receptors, mainly integrins. In this way it determines the cellular response to the biomaterial (Thomas *et al.* 1997).

Proteins that adsorb to the biomaterial surface in contact with physiological fluids (i.e., blood or cell culture media) include fibronectin, vitronectin, fibrinogen, immunoglobulins, albumin and others (Keselowsky *et al.* 2003). The type, amount and geometrical conformation of adsorbed proteins strongly depend on the physicochemical properties of the material surface, such as its chemical composition, electrical charge, wettability, roughness and topography. On polar and positively charged surfaces, e.g. those endowed with $-OH$ and $-NH_2$ groups, respectively, the spatial conformation of adsorbed fibronectin was more advantageous for binding osteoblast-like MC3T3-E1 cells through their $\alpha_5\beta_1$ integrin receptors than on non-polar and negatively charged surfaces characterized by $-CH_3$ and $-COOH$ groups, respectively (Keselowsky *et al.* 2003). The presence of polar groups results in wettability of the material surface. It has been well-established

that cells preferentially adhere to surfaces with moderate hydrophilicity (Lee *et al.* 1997, Webb *et al.* 1998). For example, the highest cell adhesion of Chinese hamster ovary cells (CHO cells) was observed on surfaces with a water drop contact angle of about 50 degrees (Lee *et al.* 1997). Good cell adhesion has been explained by the adsorption of protein molecules in an appropriate and flexible spatial conformation. This enables protein reorganization and accessibility of the specific ligands by cell adhesion receptors. On the other hand, on extremely hydrophilic surfaces, the cell adhesion-mediating proteins are bound too loosely, so they do not ensure firm adhesion and spreading of cells on the material surface (for a review, see Bačáková *et al.* 2004a, 2007a). In contrast, hydrophobic surfaces are thought to cause strong adsorption and subsequent denaturation of proteins, which distorts the conformation of cell adhesion receptor-binding domains. In addition, a preferential and strong adsorption of albumin, which acts as non-adhesive for cells, has been reported on these surfaces (Arima and Iwata 2007).

Another important factor influencing the adhesion and subsequent behavior of cells is the material surface roughness and topography. Depending on the scale of irregularities of the material surface, we can distinguish macroroughness (100 μm – millimeters), microroughness (100 nm – 100 μm), and nanoroughness (less than 100 nm), each with its specific influence. Macroroughness seems to be favourable, because it enhances the anchorage of implant into the natural tissue and is not usually felt by the cells, e.g., it does not restrict their attachment and spreading. The micro-scale roughness is more controversial, because the cells can be limited by the material surface topography in their adhesion area (Bačáková *et al.* 2001, Lossdorfer *et al.* 2004, Tan and Saltzman 2004). On the other hand, several authors have reported that osteoblasts, grown on microrough surfaces, were stimulated towards differentiation; as shown by their gene expression and higher level of mineralization in comparison with cells growing on smooth surfaces (Schneider *et al.* 2003, Lossdorfer *et al.* 2004). This disproportion can be related to the complexity of defining roughness. In the previous studies the most widely used parameter R_a was applied, which is the average peak to

valley height. This measure does not give any record of the type of surface topography; for example distances between the peaks, their sharpness, curvature of valleys etc. (Zhao *et al.* 2006, Bačáková *et al.* 2004a). The irregularities also had different shapes, e.g., pyramids, ridges, grooves, round pores, etc. Therefore it is difficult to compare the data of different research groups.

The nanoscale structure of the material surface has been found to have significant positive effects on osteoblast cell response, including initial cell adhesion and subsequent proliferation, and expression of differentiation markers. This finding is not so surprising when we keep in mind that the natural environment of cells, the extracellular matrix (ECM), is also organized in nanodimensions (Fig. 1A). Therefore many of the newly-developed bio-inspired composite materials try to mimic this effect of ECM on cells by constructing nanostructured surfaces.

The beneficial effect of the material surface nanoroughness on cell colonization has been explained by an increased amount and improved spatial conformation of the adsorbed cell adhesion-mediating proteins (Webster *et al.* 2000, Woo *et al.* 2003). Moreover, the protein adsorption was selective, showing enhancement for fibronectin and especially for vitronectin. This was attributed to a relatively small size and linear shape of the vitronectin molecule, which can conform to the nanostructure of the material better than bigger and more complicated ECM molecules, e.g., laminin (Webster *et al.* 2000). Vitronectin is recognized preferentially by osteoblasts in comparison with other osteoblast-competitive cell types. This selectivity could be highly advantageous, as it could help prevent the formation of fibrous tissue upon implantation - one of the major problems for all currently used materials - and thus lead to faster integration of the implant.

In addition, several studies have shown that for increased osteoblast adhesion the nanostructure plays a more important role than the surface chemistry. For example, in our earlier study (Bačáková *et al.* 2007b), a terpolymer of polytetrafluoroethylene, polyvinylidene fluoride and polypropylene (PTFE/PVDF/PP) was mixed with 2 to 8 wt. % carbon nanotubes, which created nano-sized irregularities on the material surface, but did not significantly change the surface

hydrophobia (the water drop contact angle about 100°). Despite this, the number and spreading of human osteoblast-like cells on the nanotube-modified surfaces was markedly increased (Fig. 2A).

The cells also require a certain level of substrate stiffness for their adhesion. During the process of adhesion and spreading, cells exert traction forces on the underlying substrate and they respond to its compliance. If the surface is too soft, as for example on polyacrylamide gels, it is not able to withstand these forces. The adhering cells are not able to spread: they are rounded, they show no assembly of cytoskeleton and focal adhesions, and consequently undergo apoptosis (Engler *et al.* 2004). Moreover, a low, medium and high level of the substrate stiffness can direct the differentiation of mesenchymal stem cells towards neuronal, muscle or osteoblast phenotype, respectively (Engler *et al.* 2006).

2.2. Integrins – major cell adhesion receptors

As mentioned above, the proteins adsorbed on the material surface in an appropriate geometrical conformation are bound by the cell adhesion receptors. The most deeply investigated and systemized group of cell-matrix adhesion receptors are integrins, i.e. heterodimeric transmembrane receptors consisting of non-covalently associated alpha and beta subunits (Hynes 2002). The extracellular domains of integrins bind to the specific amino-acid sequences in the adsorbed protein molecules, e.g. tripeptide sequence of Arg-Gly-Asp (RGD), which is the major motif in many extracellular matrix proteins; including fibronectin, vitronectin (Garcia 2005), type I collagen, osteopontin, bone sialoprotein and thrombospondin (Clover *et al.* 1992). After binding to their ligands, integrins cluster together in focal adhesions. These large supramolecular complexes contain (a) structural proteins such as talin, vinculin, paxillin and alpha-actinin, (b) signaling molecules, like focal adhesion kinase (FAK), Src and paxilin (Geiger *et al.* 2001) and (c) growth factor receptors; for example bone morphogenetic protein-2 (BMP-2) receptors (Lai and Cheng 2005). Focal adhesions, and particularly integrins, function as transmembrane structural links

between the extracellular matrix and actin cytoskeleton inside the cell (Hynes 2002). By providing the anchorage signal, all these structures directly support migration, cell cycle progression and expression of differentiation-related genes (Danen and Sonnenberg 2003).

On the osteoblast surface there are several types of integrin receptors, including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$ and $\alpha_v\beta_8$ (Clover *et al.* 1992, Gronthos *et al.* 1997). Integrins with β_1 chain seem to be the most important in osteoblast adhesion to extracellular matrix proteins including: fibronectin, type I collagen, laminin and vitronectin (Gronthos *et al.* 1997), and play a major role in osteoblast differentiation (Schwartz *et al.* 2007). The osteoblast differentiation is manifested by numerous specific markers, the most followed of them being the synthesis of calcium-binding glycoproteins osteocalcin and osteopontin, collagen I, the activity of alkaline phosphatase and the bone tissue mineralization (Losdorfer *et al.* 2004, Kesselovski *et al.* 2005, Lai and Cheng 2005, Sun *et al.* 2006, Inanc *et al.* 2007, Dadsetan *et al.* 2008, Marie 2008, Müller *et al.* 2008, Satija *et al.* 2007). Alpha_v integrins also stimulate the osteoblast differentiation by interacting with receptors for BMP-2 (Lai and Cheng 2005), although $\alpha_v\beta_3$ integrins were reported to retard the osteoblast differentiation and bone matrix mineralization, while the cell proliferation was enhanced (Cheng *et al.* 2001).

It should also be noted that, although integrin receptors are recognized as the major class of cell adhesion receptors to extracellular matrix, some studies have shown that other, non-integrin receptors also take part in this process. For example, heparan sulphate proteoglycan on osteoblasts has been found to recognize a bone-specific oligopeptide Lys-Arg-Ser-Arg (KRSR) (Dee *et al.* 1998). However, the cell-matrix adhesion (mediated by non-integrin adhesion receptors) is not still fully understood (Bačáková *et al.* 2004a).

3. Materials used or tested for bone implants

3.1. Single-phase materials

Currently used and tested materials designed for the construction of bone implants and replacements particularly include metallic alloys, ceramics and synthetic polymers. All of these materials have certain advantageous properties, but all of them have been proven to also possess negative characteristics which limit their widespread use.

Most of the implants currently utilized in bone surgery are metallic alloys containing Co, Cr, Mo, Ni or Ti. Metals were chosen as suitable materials thanks to their good mechanical properties, e.g., stiffness, which makes them especially suitable for load-bearing implants. However, they do not match the mechanical properties of natural bone, as they are more rigid and weighty. This stimulates remodeling of the surrounding bone tissue by resorption, because the strain or stress imposed on the bone is carried particularly by the stronger implant. Consequently this phenomenon in the long term causes aseptic loosening of the implant (Wang 2003). Another risk associated with the use of metallic implants is that in the environment of body fluids they undergo corrosion and they release metallic ions which are cytotoxic or immunogenic in higher concentrations (Park and Kim 2003).

By contrast, ceramics are in general highly biocompatible. Some, e.g., hydroxyapatite, are even strongly bioactive and are able to form a direct bond with the bone tissue. However, their major shortcoming is their insufficient elasticity for use in bone implants, as they are susceptible to cracking and breaking (Billotte 2003). Polymeric materials provide enormous variability in their properties. Currently used polymers are all biocompatible and light, however they are too soft and elastic, and are not able to carry the weight load on their own.

3.2. Multi-phase materials (composites)

Composite materials, by definition, are materials consisting of two or more different constituents at a micro- or macroscale range having a distinct interface separating them. Their major advantage is that they offer the possibility of combining properties of the initial material to engineer a new construct which would have desirable properties distinct from the properties of the original materials. This approach is now widely used in constructing novel biomaterials for bone implants, taking inspiration from natural bone tissue, which is itself a natural composite.

The mechanical properties of the composite material depend not only on the type of combined materials, but also on the volume fraction and shape of the heterogeneities (particles, fibers, whiskers, platelets, etc.), according to which they are classified into certain groups (Lakes 2003). In the field of biomaterials, fiber- or particle-reinforced composites are of special interest. Usually the harder or stronger phase of the composite is discontinuous and forms the reinforcement, and it is embedded in a continuous phase referred to as matrix (Migliarese and Alexander 2004). This kind of organization of the composite partially follows the hierarchical architecture of natural bone, which is basically a collagen-hydroxyapatite composite (Wang 2003; Fig. 1A). In the ECM of bone tissue, the collagen type I fibers provide the strength and function as the reinforcement. These fibers are embedded in a matrix made of other proteins or proteoglycans (such as osteocalcin, osteopontin, osteonectin, bone sialoprotein and bone morphogenetic proteins), which play important roles in controlling the function of osteoblasts, as well as bone tissue mineralization (Inanç *et al.* 2007, Satija *et al.* 2007, Dadsetan *et al.* 2008, Marie 2008, Müller *et al.* 2008). Both fibers and bone matrix are closely associated with the inorganic particulate component of the bone, i.e., crystals of hydroxyapatite and other calcium phosphates, mainly tricalciumphosphate. Therefore, in the construction of so called “bio-inspired” materials for bone implants, materials consisting of a polymer matrix containing a particulate, bioactive component seem to be the natural choice (Wang

2003). Polymer matrix can be further reinforced by fibers that would, similarly as collagen, strengthen the whole construct.

As mentioned above, the performance of the composite materials could be markedly improved if these materials are constructed in the form of three-dimensional scaffolds (Fig. 1B). If these cell carriers are degradable, they can be gradually replaced with regenerated bone tissue. The ingrowth and maturation of cells inside the scaffolds could be significantly stimulated by dynamic cell cultivation in perfusion or rotational bioreactors. In comparison with classical static cell culture systems, these bioreactors ensure a better supply of oxygen and nutrient to the cells, quicker waste removal, and provide the cells with mechanical stimulation, which is beneficial for their differentiation and functioning (Janssen *et al.* 2006, Buzcynska *et al.* 2007, Zhao *et al.* 2007). Similar favorable effects on the bone tissue formation were also induced using an electromagnetic bioreactor (Fassina *et al.* 2007).

3. 2. 1. Fibrous component of the composites

The fibrous component of the composites designed for the bone implantation has often been created from natural and synthetic polymers, bioglass, carbon or combinations of these materials. The natural polymers have usually been represented by collagen (Shih *et al.* 2006, Venugopal *et al.* 2008), gelatin (Casper *et al.* 2007), chitosan (Kong *et al.* 2005, Bhattarai *et al.* 2005) or silk fibroin (Li *et al.* 2006). However, their synthetic counterparts involved a wide range of degradable and stable polymers, such as polycaprolactone (Wutticharoenmongkol *et al.* 2007); polylactide, polyglycolide and their copolymers (Kim *et al.* 2006, Buzcynska *et al.* 2007, McCullen *et al.* 2007, Jeong *et al.* 2008); polyphosphazenes, a class of special inorganic-organic polymers known for high biocompatibility, high-temperature stability, and low-temperature flexibility (Nair *et al.* 2004); and aliphatic or aromatic polyamides, such as Aramide (Baidya *et al.* 2001, Bačáková *et al.* 2007c; Balík *et al.* 2008; Fig. 2C, D). Also, composites with a carbon or polymer matrix reinforced with

carbon fibers (Fig. 2B) have long been considered to be very promising for bone tissue implantation and replacement, due to their excellent mechanical properties as well as biocompatibility (Sagomyants *et al.* 2008). However, there has been some concern about the fact that in some *in vitro*, as well as *in vivo* studies, they have been shown to release small particles and debris (Lewandowska-Szumiel *et al.* 1999, Bačáková *et al.* 2001).

For advanced composites, fibers of nano-sized diameter have usually been applied. For example, bioglass nanofibers, in a polylactide matrix, induced rapid spontaneous formation of a hydroxycarbonate apatite layer in a simulated physiological medium (i.e., with ion concentrations similar to those in the human body plasma), as well as maturation of osteoblasts (Kim *et al.* 2008). Polymeric nanofibers have been loaded with growth factors, mainly bone morphogenetic proteins (Li *et al.* 2006, Park *et al.* 2006) or ceramic and carbon nanoparticles in order to increase their bioactivity and mechanical strength. For example, hydroxyapatite nanoparticles or carbon nanotubes were encapsulated inside collagen or polylactide nanofibers without forming lumps on the nanofibre surface (Venugopal *et al.* 2008, Jeong *et al.* 2008, McCullen *et al.* 2007). Also the carbon nanotubes themselves were used for reinforcement of synthetic polymers or chitosan. These nanoparticles can resemble nanofibers of collagen and other extracellular proteins of the bone, as well as hydroxyapatite and other inorganic crystals in the bone (Price *et al.* 2004). As a component of polymeric porous scaffolds for bone tissue engineering, they can form nano-sized irregularities on the pore walls and thus promote the ingrowth of bone cells inside the material (Woo *et al.* 2003, Tan and Saltzman 2004). In addition, carbon nanotubes can be used for electrical stimulation of osteoblasts, which has been reported to promote their proliferation, differentiation, production of mineralized bone matrix, and thus healing of the damaged bone (Supronowicz *et al.* 2002, Zanello *et al.* 2006, for a review, see Bačáková *et al.* 2008). When released from degradable polymeric scaffolds, carbon nanotubes could be relatively quickly eliminated from the organism by glomerular filtration (McDevitt *et al.* 2007). On the other hand, there is a considerable risk of immunogenic or

even genotoxic, mutagenic and carcinogenic action of carbon nanotubes (Zhu *et al.* 2007, Herzog *et al.* 2007, Chou *et al.* 2008; for a review, see Bačáková *et al.* 2008). Sato and Webster (2004) warn about some of the concerns associated with the use of nano-particles of all kinds in the human body, as they may possibly be released from the implant, and their effect on human health is still unknown.

3. 2. 2. Matrix component of the composites

Similarly to the fibers, the matrix of artificial bone replacements has usually been made of natural or synthetic polymers; the latter being degradable or durable. Examples of promising and interesting matrices include gelatin, chitosan, alginate (Ren *et al.* 2002, Li *et al.* 2005), hyaluronic acid (Bakos *et al.* 1999), polylactides and their copolymers with glycolides (Kim *et al.* 2008, Obata and Kasuga 2008, Pamula *et al.* 2008), hydrogels based on poly(ethylene glycol), PEG (Dadsetan *et al.* 2008, Jung *et al.* 2008) or macroporous hydrogels such as poly(2-hydroxyethylmethacrylate), poly(HEMA) (Lesný *et al.* 2006), siloxane-based materials (Ren *et al.* 2002, Bačáková *et al.* 2007c, Obata and Kasuga 2008) polyetheretherketone, PEEK (Baidya *et al.* 2001, Sagomonyants *et al.* 2008), poly (ethylene terephthalate), PET (Zhao *et al.* 2007), polyurethanes (Fassina *et al.* 2007), polyamides (Wang *et al.* 2007), high density polyethylene, HDPE (Homaeigohar *et al.* 2008) as well as various combinations of these materials, e.g., gelatin-siloxane or chitosan-alginate hybrids (Ren *et al.* 2002, Li *et al.* 2005) or siloxane-containing poly-(lactic acid) composites (Obata and Kasuga 2008). Polytetrafluoroethylene (PTFE) has been applied in the form of membranes for stimulation of bone tissue regeneration in oral or craniofacial applications (Lilli *et al.* 2002, Suzuki *et al.* 2005). All the mentioned materials have often been combined with inorganic particles and loaded with bioactive molecules, such as bone morphogenetic protein-2 or transforming growth factor beta-1 (Lilli *et al.* 2002, Wang *et al.* 2007, Homaeigohar *et al.* 2008, Jung *et al.* 2008, Kim *et al.* 2008, Obata and Kasuga 2008).

3. 2. 3. *Inorganic particulate component of the composites*

As the particulate component of the “bio-inspired” composites, hydroxyapatite (HAp) particles have been widely used. This ceramic material belongs to a large group of calcium phosphates. HAp has several advantages, as mentioned above. First of all, it is highly biocompatible and bioactive. It is able to form strong bonds with the bone tissue and conduct bone formation. Modification of the material surface with HAp was shown to be stimulatory for cell proliferation (Vagaska *et al.* 2006). The underlying mechanism of its high biocompatibility lies in its ability to adsorb cell adhesive proteins (especially fibronectin and vitronectin) from the serum, which in turn enables osteoblast adhesion through integrin receptors (Kilpadi *et al.* 2001, Woo *et al.* 2007).

Recently, with the increasing interest in nanotechnologies, nanoparticles of HAp similar to those in the extracellular matrix are used. This approach has several advantages. First, nanoparticles have the ability to improve the mechanical properties, e.g., to increase the strength of the composite (Kim *et al.* 2006, Wang *et al.* 2002, Ramay and Zhang 2004, Huang *et al.* 2007), especially in porous scaffolds that cannot yet be used in load-bearing implants. In addition, nano-HAp has been proven to have positive effects on cell-biomaterial interactions (Webster *et al.* 2000). For example, HAp nanoparticles evenly dispersed on the pore walls in a porous chitosan composite enhanced spreading and proliferation of osteoblasts in comparison with scaffolds without HAp (Kong 2005). Similarly, HAp nanoparticles exposed on the surface of poly(lactide-*co*-glycolide) scaffolds (i.e., not covered by the polymer matrix) increased the cell numbers, level of cell differentiation and matrix mineralization (Kim *et al.* 2006). Therefore, increased bone tissue formation can be directly related to the contact of osteoblasts with nano-HAp particles. It has been even suggested that the positive effects of HAp nanoparticles on the osteoblast behavior is comparable with the effect of functionalization of ceramics with synthetic RGD sequences (Balasundaram *et al.* 2006). This also implies an immense influence of the nanotopography of new biomaterials in general.

However, at this point it should also be mentioned that not only the size of the HAp particles, but also their composition, crystallinity and shape are important in order to have a stimulatory effect on the cell. Chou *et al.* (2005) examined the effect of five different types of apatite particles, which had different effects on the cell viability, proliferation as well as gene expression and differentiation. For example, particles with a less stable crystal structure dissolved more rapidly and caused a local increase of Ca^{2+} ions in the microenvironment, which induced apoptosis of the seeded osteoblasts. The cells growing on different types of particles showed a different morphology, and in general they had a lower proliferation and adhesion area in comparison with the control cells growing on the tissue culture polystyrene dish, probably due to the microsize of some of the types of HAp.

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 the 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 (Yamada *et al.* 1997, John *et al.* 2003, Detsch *et al.* 2008). In our earlier study performed in conventional static cell culture system, adhesion substrates for human osteoblast-like MG63 cells, made of beta-TCP, induced strong alkalization of the cell culture media, followed by cell death (Bačáková *et al.* 2004b).

In addition to calcium phosphates, other inorganic compounds have been used in order to enhance the bioactivity of artificial supports for bone tissue reconstruction, such as calcium carbonate, silicate, sulphate or oxide (Verne *et al.* 2005, Guo *et al.* 2007, Cui *et al.* 2008, Obata and Kasuga 2008), phosphates, carbonates, silicates and oxides of magnesium, sodium or potassium (Gough *et al.* 2003, Bačáková *et al.* 2004b, Ramaswamy *et al.* 2005, Verne *et al.* 2005, Sun *et al.* 2006, Knabe *et al.* 2008, Ponader *et al.* 2008) barium sulphate (Ricker *et al.* 2008) or molecules

containing fluorine or zirconium (Ramaswamy *et al.* 2005). Similarly as the fibers and matrix molecules in the composites, the inorganic particulate component of the material can also be loaded with various growth factors or drugs stimulating bone tissue regeneration (Cui *et al.* 2008, Ponader *et al.* 2008).

4. Conclusion

Biomaterials constructed for bone replacements have to meet specific requirements for mechanical properties, biocompatibility and bioactivity. They should mimic the hierarchical architecture or chemical composition of the bone.

Single-phase materials, such as metals, ceramics, and polymers do not possess optimal properties. Metal alloys are too rigid and weighty, metallic ions released are cytotoxic, ceramics are not elastic and crack easily, synthetic polymers are too soft, and are not able to carry the weight load.

Novel multi-phase composite materials, constructed for bone implants, take inspiration from natural bone tissue, which is itself a natural collagen-hydroxyapatite composite. Their major advantage is that they offer the possibility to combine properties of the initial material, in order to engineer a new construct, with desirable properties distinct from the properties of the original materials. The mechanical properties of the composite material depend not only on the type of combined materials, but also on their volume fraction and shape. In bone tissue replacements, fiber- or particle-reinforced composites are of special interest. A stronger discontinuous phase of the composite forms the reinforcement that is embedded in a continuous phase referred to as matrix. Both fibers and bone matrix are closely associated with the inorganic particulate component of the bone, i.e. crystals of hydroxyapatite and other calcium phosphates. Calcium phosphates belong to bioactive molecules which have been observed to improve material integration in an organism and bone regeneration. Bioactivity is also connected with nanorough surfaces, which support protein

adsorption and preferential adhesion of certain cell types; many types independently of chemical composition. The effect of nanostructures on cell growth is comparable to other highly advanced strategies, such as incorporating synthetic ligands for cell adhesion receptors (e.g., RGD or KRSR sequences) onto the material surface, construction of three-dimensional materials with a controllable degradation rate, or controlled release of growth factors and drugs from these materials.

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7. Figures

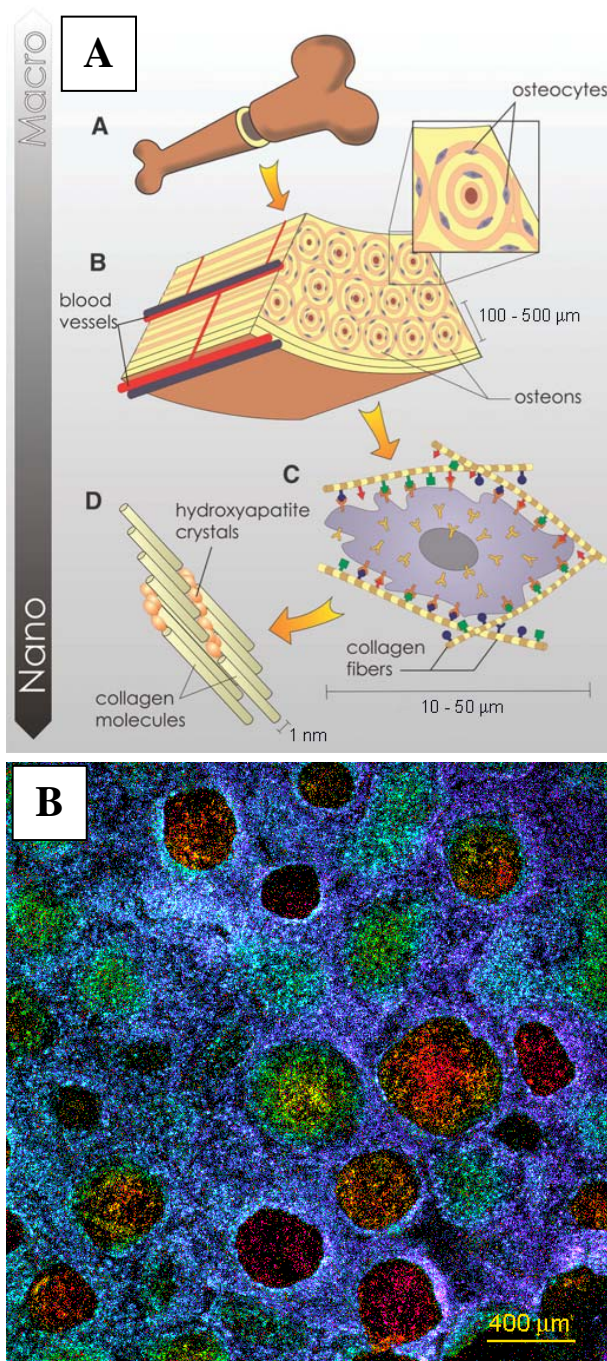


Fig. 1. A. Hierarchical organization of bone on different size scales, including nanoarchitecture of the extracellular matrix (Stevens and George 2005). **B.** Imitation of this structure by an experimental bioartificial bone construct containing a porous poly(L-lactide-*co*-glycolide) scaffolds and human osteoblast-like MG 63 cells. The depth of cell ingrowth into the pores is indicated by spectral colors (blue: depth of 0–60 μm , green: 80–160 μm , yellow: 180–220 μm , orange: 240–300

μm , red: 320–400 μm , violet: 420–480 μm). Day 14 after seeding, cells stained with propidium iodide. Leica TCS SP2 confocal microscope (Pamula *et al.* 2008).

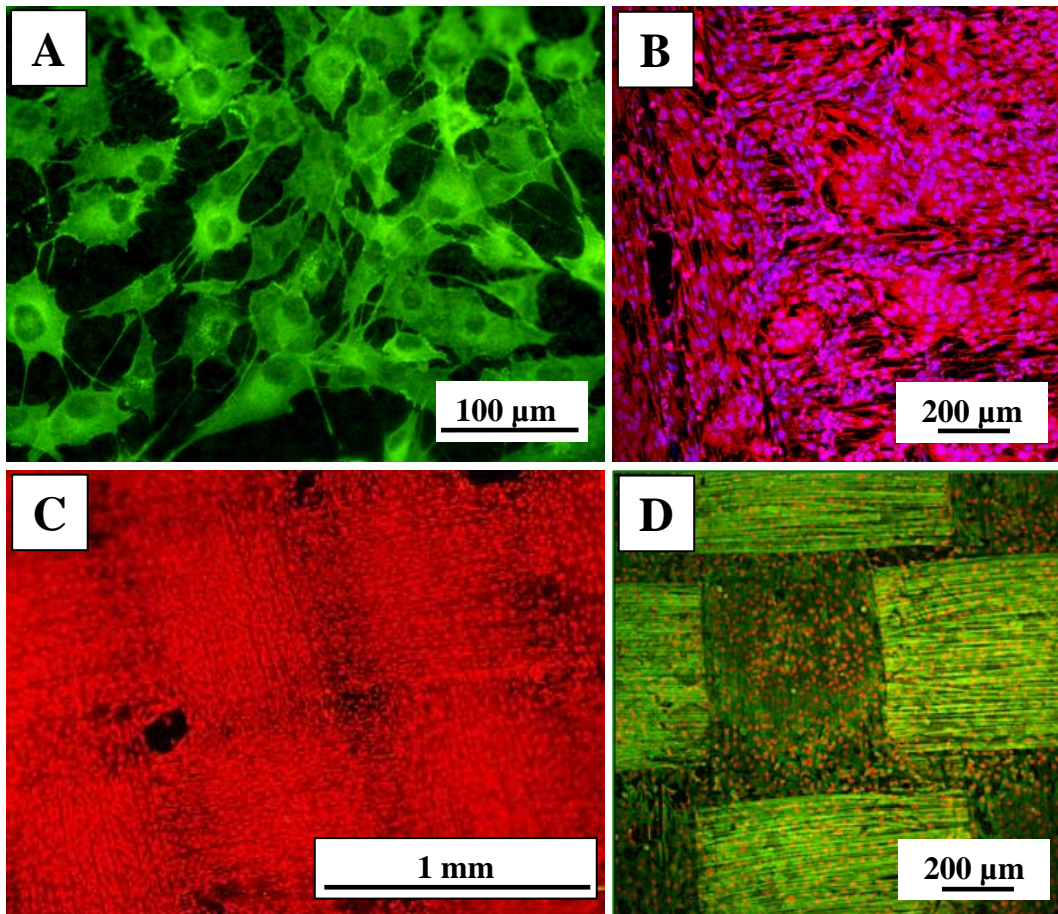


Fig. 2. Human osteoblast-like MG 63 cells in cultures on a terpolymer of polytetrafluoroethylene, polyvinylidene fluoride and polypropylene with 4 wt.% of multi-wall carbon nanotubes (**A**), a composite with the carbon matrix reinforced with carbon fibers (**B**), Aramide fabrics (**C**) and a composite with polysiloxane matrix reinforced with Aramide fabrics (**D**). **A**: immunofluorescence of β -actin, **B**: Hoechst # 33342 and Texas Red C₂ maleimide, **C**: propidium iodide, **D**: propidium iodide and immunofluorescence of β -actin. Day 3 (**A**, **D**) or day 7 (**B**, **C**) after seeding. **A**, **C**: Olympus IX 51 microscope, DP 70 digital camera; **B**, **D**: Leica TCS SP2 confocal microscope. Bar: 100 μm (**A**), 200 μm (**B**, **D**), 1 mm (**C**).