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Human osteoblast-like cells in cultures on polymer based composite materials developed for bone implants

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Abstract

Novel polymer-polymer based composites for load-bearing bone implants are being developed. By partially mimicking the natural architecture of bone tissue they are able to meet the necessary mechanical requirements. However, the new generation of implants should also be bioactive, which means that the implants should elicit specific desired cellular responses leading to integration of the material into the natural tissue and stimulating self-healing processes.

One of the strategies applied is to modify the physico-chemical properties of the material surface, such as surface chemistry, wettability or roughness, which have previously been shown to have a substantial influence on the cell-biomaterial interaction. In recent years, considerable attention has been paid to the potential use of nanobiotechnology. Nanostructured biomaterials have namely been shown to have a stimulatory effect on osteoblast proliferation and differentiation. The underlying mechanism seems to be a change in the surface energetics, leading to increased and selective adsorption of cell adhesion-promoting proteins like fibronectin and vitronectin. Nanoscale architecture also induces a favourable conformation, enhancing the availability of specific integrin-binding domains for the cell. Creating nanorough surfaces seems to be a very promising strategy for functionalizing biomaterials, as the positive effect of such surfaces appears to be independent of the material type. Moreover, they selectively stimulate osteoblasts in comparison with other competitive cell types, such as fibroblasts, which could prevent fibrous tissue formation upon implantation.

Based on these findings, a novel composite material is being developed from polyamide fiber reinforcement embedded in polysiloxane matrix. Bioactivity should be enhanced by hydroxyapatite particle inclusions. In the experimental part of this work, the biocompatibility of the reinforcing fabrics and of the first composite constructs was tested. The results have shown that these materials are able to support osteoblast-like cell adhesion and proliferation and after some modifications (especially decreasing the size of the HAp particles to nanodimensions) could be promising for future use in hard tissue surgery.

Key Words: bone tissue engineering, polymer-based composites, non-resorbable composites, hydroxyapatite particles, nanotechnology, nanoroughness, osteoblasts, bioartificial bone

Abstrakt

V súčasnosti prebieha vývoj nových kompozitných materiálov pre kostné implantáty, ktoré sú založené na kombinácii polymérnej výstuže a polymérnej matrici. Čiastočným napodobňovaním prirodzenej architektúry kostného tkaniva sú schopné splniť požiadavky na potrebné mechanické vlastnosti. Nová generácia implantátov by však mala byť aj bioaktívna, to znamená, že by mala byť schopná vyvolať požadovanú špecifickú bunečnú odpoveď, ktorá by viedla k lepšej integrácii materiálu do prirodzeného kostného tkaniva a stimulovala by proces samo hojenia.

Jedna z aplikovaných stratégii k dosiahnutiu tohto cieľa zahŕňa modifikácie fyzikálno-chemických vlastností povrchu materiálu, napríklad povrchového chemického zloženia, zmáčavosti alebo drsnosti, ktoré podstatne ovplyvňujú interakciu bunky s umelým materiálom. V posledných rokoch sa značná pozornosť sústredila najmä na potenciálne využitie nanobiotechnológií. Zistilo sa, že nanoštrukturované biomateriály majú stimulačný efekt na proliferáciu a diferenciáciu osteoblastov. Mechanizmus tohto efektu sa zdá byť v zmenenej povrchovej energetike a následnej selektívnej adsorpcii adhezivných proteínov extracelulárnej matrix ako fibronektínu a vitronektínu. Zároveň, povrchová architektúra v nanorozmeroch indukuje ich výhodnú konformáciu tým, že zvyšuje mieru dostupnosti špecifických integrín-viazajúcich domén pre adherujúcu bunku. Vytváranie nanodrsných povrchov sa javí ako veľmi nádejná metóda ako funkcionalizovať biomateriály, keďže ich pozitívny efekt vyzerá byť nezávislý na type materiálu. Okrem toho sú preferenčne stimulované osteoblasty v porovnaní s inými kompetitívnymi bunečnými typmi, napr. fibroblastmi, čo by mohlo preventívne pôsobiť proti vzniku fibrózneho tkaniva v okolí implantátu brániaceho pevnej väzbe s prirodzenou kosťou.

Na základe týchto zistení je konštruovaný aj nový kompozitný materiál založený na polyamidovej vláknitej výstuži a polysiloxanovej matrici. Bioaktivita materiálu by mala byť zvýšená hydroxyapatitovými časticami. V experimentálnej časti bola hodnotená biokompatibilita vystužujúcej tkaniny ako aj prvých kompozitov. Výsledky ukazujú, že tieto materiály umožňujú adhéziu a proliferáciu kostných buniek a po určitých úpravách (zníženie veľkosti hydroxyapatitových častíc na nanoveľkosť) by mohli nájsť využitie v chirurgii.

Kľúčové slová: tkanivové inžinierstvo kosti, polymérne kompozity, neresorbovateľné kompozity, hydroxyapatitové častice, nanotechnológie, nanodrsnosť, osteoblasty

CONTENTS

1. Introduction

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

The main requirement apart from mechanical properties that is imposed on these implanted artificial materials, or biomaterials, 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 have 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 (Bacakova *et al.* 2004, Hench and Polak, 2002).

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, which 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 (Bacakova *et al.* 2004). 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).

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, in the first part of my thesis I will introduce what is already known about this mechanism and what factors and material properties may have a significant influence on it. In the second part, I will talk about the newly developed polymer-based composite materials that are constructed to mimic the natural organization of bone, with special reference to the composite newly developed in our laboratory. And finally, in the experimental part of this work I will report on some of the first results in biological testing of these materials, and possible future research.

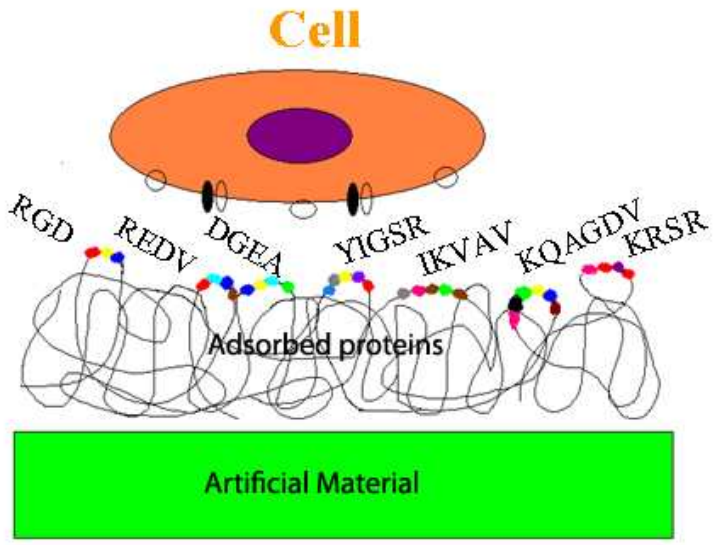
2. Interaction between the material and bone cells

As it was stated above, understanding the interface of the cell and the artificial material has been one of the major challenges in recent biomaterials research. Satisfactory comprehension of this phenomenon would namely enable us to engineer the material surface in a way that would elicit the desired cellular response, e.g., proliferation and differentiation, and suppress the effects that impair its function, especially adverse immune responses.

Immediately after the biomaterial is implanted or comes into contact with physiological fluids, protein adsorption to its surface occurs. This happens within one second, 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 its surface receptors, integrins (Fig. 1). In this way it determines the cellular response to the biomaterial (Thomas *et al.* 1997).

Fig. 1. Mechanism of cell adhesion to artificial materials.



2.1. Protein adsorption

Proteins that adsorb to the biomaterial surface in contact with physiological fluids include immunoglobulins, vitronectin, fibrinogen, fibronectin, albumin and others (Keselowsky *et al.* 2003).

Adsorption kinetics can be divided into three distinct phases. During the initial rapid phase it is influenced only by diffusion rate, and the increase in weight of the adsorbed proteins is almost linear when plotted against time. This phase is very quick. Within one second, 50% of all the proteins present in the final phase have already adsorbed. Later, during the second phase, due to the competition for empty places the rate slows down. Finally, in the steady phase, the final amount of proteins is more or less constant. This was measured to be about 0.1-0.5μg/cm^2 depending on the size and conformation of the proteins (Horbett 2004). This kinetics and the existence of competition among the proteins for a limited number of empty sites available for direct binding to the materials surface support a monolayer model, which predicts only one tightly packed layer of strongly attached proteins.

The composition and fractional representation of the adsorbed proteins does not have to be the same as in the bulk of the fluid from which they adsorb. This so-called enrichment is also one of the results of competition among proteins. It depends not only on the concentration in the bulk

solution, but also on the affinity of the proteins, which in turn depends on the surface properties (Horbett 1999).

Adsorbed proteins are strongly bound to the surface by multiple non-covalent interactions, and they can be removed only by a detergent. However, their composition in time is not constant, and they may be exchanged with other proteins in solution (the so-called Vroman effect; Jung *et al.* 2003). A change in composition may also occur after the adhesion of cells that can actively reorganize the underlying substrate and deposit their own extracellular matrix proteins.

Another important consequence of protein adsorption to the material surface is the change in the conformation of the molecules. This is the result of protein intrinsic instability and a tendency to unfold in order to increase the binding area. The change in the conformation depends on the surface properties, and it modulates the functional presentation of the adsorbed proteins to cells receptors, and consequently it may have a significant effect on the cell response (Horbett 2004).

2.2. Integrins – major adhesion receptors

The understanding of the molecular mechanisms underlying different cellular reactions to synthetic surfaces still remains incomplete. However, it has been well established that integrin adhesion receptors act as the central regulators of cell-biomaterial interaction (Garcia 2005).

Integrins are heterodimeric transmembrane receptors consisting of non-covalently associated alpha and beta subunits (Fig. 2). Their extracellular domains bind to the components of the extracellular matrix or the adsorbed layer of proteins. Currently, 8 beta and 18 alpha subunits are known that combine to form 24 distinct integrin receptors. According to the ligand that they bind to, they can be divided into 3 sets. For adhesion to biomaterials the most important group of receptors is the group that recognizes the 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). The two other sets include laminin receptors and integrins that recognize Ig-superfamily cell surface counterreagents (Hynes 2002).

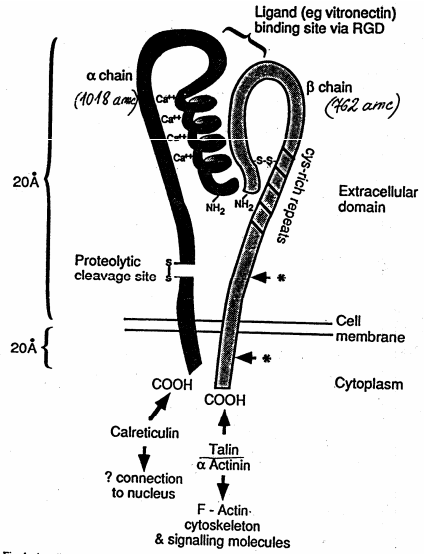


Fig. 2. Structure of the integrin receptor. (Reproduced from: Horton 1997)

After binding to their ligands, integrins have a tendency to associate with the actin skeleton and cluster together in focal adhesions. These large supramolecular complexes contain:

a) structural proteins such as vinculin, talin and alpha-actinin
b) signaling molecules like FAK (focal adhesion kinase), Src and paxilin (Geiger *et al.* 2001)
c) growth factor receptors, for example BMP-2 receptors (Lai and Cheng 2004)

Focal adhesions and particularly integrins function as transmembrane structural links between the extracellular matrix and cytoskeleton inside the cell (Hynes 2002; Fig. 3). By providing the anchorage signal, all these structures directly support migration, cell cycle progression and expression of differentiation-related genes (Danen and Sonnenberg 2003). In this so-called outside-in signaling, integrins modulate various signal transduction cascades similar to those triggered by growth factor receptors, and in many cases these pathways are even closely coupled (Hynes 2002).

As noted by Hynes (2002), integrins act as bidirectional signaling machines that are able to signal also in the opposite inside-out direction by modulating their function and affinity for ligands from within the cell.

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 β_1 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). Alpha$_V$ integrins play an important role in signaling and osteoblast differentiation by interacting with growth factor receptors (Lai and Cheng 2004).

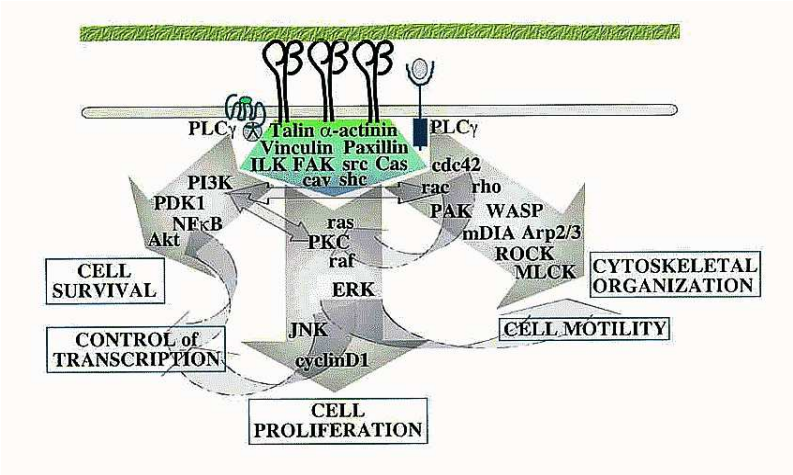


Fig. 3. Signaling through integrin receptors. Pathways leasing to major effects on cell behaviour are summarized (from Hynes 2002)

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 these adhesion receptors is not still fully understood (Bacakova *et al.* 2004).

2.3. Physical and chemical surface properties of the material that influence cell response

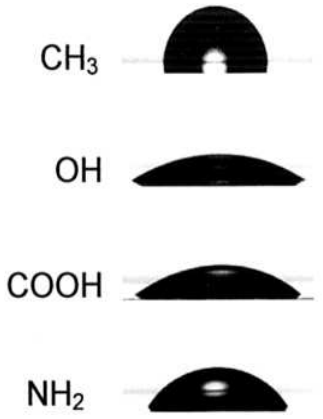
As was mentioned above, the type, quantity and conformation of adsorbed proteins having a profound effect on the cellular response depend mainly on the material surface properties. These include especially surface chemistry, which in turn affects wettability and the surface charge, as well as physical properties, such as surface topography, roughness and rigidity. In the following section I will comment on their effects independently, but it should borne in mind that these are simplified models and under real conditions all of these factors influence the cell behaviour simultaneously in a more complex way.

2.3.1. Surface chemistry

One of the methods for studying the effect of the surface chemistry is to prepare chemically well-defined surfaces followed by observing adhesion of a single type of protein and subsequent cell adhesion and other responses. Keselowsky *et al.* (2003) observed the adsorption of fibronectin and the subsequent response of osteoblast-like cells to self-assembled monolayers (SAMs) of alkanethiols ($HS–(CH_2)_n–X$; $n \geq 10$). In their study they tested 4 different types of surface chemistry given by the functional –X groups: $–CH_3$ as a representative of non-polar surface; –OH as a neutral polar group; –COOH, as negatively charged and $–NH_2$ positively charged groups under the

physiological pH=7.4. The surface charge and wettability of the samples characterized by water contact angle measurements are shown in Fig. 4. As expected, the different surface chemistry resulted in different fibronectin adhesion and conformation. Monoclonal antibody studies against the central binding domain on fibronectin showed differences in their affinity in the following pattern: OH > COOH = NH_2 > CH_3.

Fig. 4. Principle of the measurement of water drop contact angle on the material surface. (Reproduced from Keselowsky *et al.* 2003)



	Contact Angle
CH_3	107 ± 1°
OH	25 ± 3°
COOH	28 ± 1°
NH_2	43 ± 3°

The central domain consists of 9th type III repeat with RGD sequence and 10th type III repeat with PHSRN sequence, both of which are crucial for classical fibronectin integrin ($\alpha_5\beta_1$) binding. Moreover, not only the presence of these motifs, but also their relative spatial orientation plays an important role in the recognition process. Therefore any conformational changes in these domains induced by the material surface may have a significant effect on the cell integrin binding and the subsequent cell response (Grant *et al.* 1997).

This was also proved in the above-mentioned study. Binding of $\alpha_5\beta_1$ integrin followed the same trend OH > COOH = NH_2 > CH_3. The same result was obtained in osteoblast-like cell adhesion strength, with the highest strength on a neutral polar surface and the lowest on a non-polar hydrophobic surface that strongly denatures adsorbed proteins (Keselowsky *et al.* 2003).

The consequent effects of this phenomenon were observable on cell focal adhesion composition and signaling through integrins (Keselowsky *et al.* 2004), as well as on cell differentiation (Keselowsky *et al.* 2005, Garcia *et al.* 1999). Surfaces with the –OH functional group exposed supported the highest levels of recruitment of structural proteins (talin and α-actinin) and signaling molecules (paxilin and tyrosine-phosphorylated proteins) to focal adhesions. The lowest levels were again observed on surfaces covered with the –CH_3 functional group, which

strongly correlated with $\alpha_5\beta_1$ integrin binding (Keselowsky *et al.* 2004). Phosphorylation of specific Tyr residues on FAK (major kinase at focal adhesions that activates several pathways regulating cell survival, proliferation, etc.) also followed a similar trend (NH_2 >OH = COOH>CH_3). Higher osteoblast differentiation and matrix mineralization was observed on –NH_2 and –OH surfaces, and low values in the case of –COOH and –CH_3. As was mentioned above, the non-polar methyl group is hydrophobic, causing denaturation of proteins. Carboxyl group surfaces, on the other hand, selectively bind $\alpha_V\beta_3$ integrins, which inhibit prodifferentiation signals triggered by $\alpha_5\beta_1$ integrins (Keselowsky *et al.* 2005). Studies like these show that the single protein adsorption method can clarify quite complex details about the mechanism of cell-material interaction, inducing cell adhesion and differentiation.

However, some authors argue that this approach does not mimic what happens in reality at the implant interface, which is exposed to a solution of many proteins at the same time. For example, studies with selective depletions of a single protein from the mixture (e.g. Thomas *et al*. 1997) have shown that vitronectin has a much more influential (regulatory) effect on initial cell adhesion than the above-mentioned fibronectin. Fibronectin-depleted serum had very little effect on cell culture in comparison with the sample where full-serum was added. However, when vitronectin was depleted (both alone and together with fibronectin) the cell adhesion and spreading was severely impaired. Only 5% of the seeded cells attached and even those were not well spread, mostly round-shaped. Due to these results, the authors place great emphasis on studies with complex media to give a more realistic view of what is happening at the cell-biomaterial interface.

2.3.2. Wettability

The chemical functional groups exposed on the surface of the material affect predominantly its wettability. It has been well-established that cells preferentially adhere to surfaces with moderate hydrophilicity, as reported by several groups of authors and for different cell types (Lee *et al*. 1997, Webb *et al.* 1998). For example Lee *et al.* (1997) created functional group gradients to test a wider range of wettability while other factors (especially surface charge) remained unchanged. The highest cell adhesion of Chinese hamster ovary cells (CHO cells) was observed on surfaces with a contact angle of about 50 degrees. Good cell adhesion on moderately hydrophilic surfaces has been explained by adsorption of protein molecules in an appropriate and flexible spatial conformation enabling protein reorganization and accessibility of the specific ligands by cell adhesion receptors (for a review, see Bacakova *et al.* 2004, 2007). On the other hand, on extremely hydrophilic surfaces, the cell adhesion-mediating proteins are bound too loosely (weakly), so they do not ensure

firm adhesion and spreading of cells on the material surface (for a review, see Bacakova *et al.* 2004, 2007).

Hydrophobic surfaces, as mentioned above, are thought to cause strong adsorption and subsequent denaturation of proteins, which distorts the conformation of cell adhesion binding domains. In addition, a preferential and strong adsorption of albumin, which acts anti-adhesively for cells, has been reported on these surfaces (Arima and Iwata 2007).

2.3.3 Surface charge

The type of functional groups exposed on the surface also influences the overall surface charge. It has been reported by several authors that cells prefer a positively charged surface to a negatively charged surface (e.g. with $-NH_3$ groups exposed) (Lee *et al*. 1997, Keselowsky *et al*. 2005). Lee explains this preference by the fact that a large proportion of serum proteins that mediate cell adhesion are recognized as negatively-charged, and this induces protein interaction with the surface.

2.3.4. Substrate rigidity

In order to complete the list of factors that influence cell adhesion in general, it should be mentioned that cells require a certain level of substrate stiffness. 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 polyacryalamide gels, it is not able to withstand these forces and 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). However, as the materials for bone tissue engineering are constructed as load-bearing, they are sufficiently stiff and this factor is not of much importance in this case.

2.3.5. Surface roughness and topography

Not only surface chemical properties, but also topography and roughness have been shown to have a significant effect on protein adsorption and the subsequent cell behavior. Depending on the scale of irregularities of the material surface we can distinguish macro- (100μm – milimeters), micro- (100nm – 100μm), 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 felt by the cells, e.g. it does not restrict their adhesion and spreading.

2.3.6. Microroughness

Cells typically between 10-100μm in size are inherently sensitive to the microtopography of their environment (Stevens and George 2005). It has been reported that microroughness significantly affects cell response to the material. For example Schneider *et al.* (2003) and Lossdorfer *et al.* (2004) have shown 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. On the other hand, several authors have reported decreased cell proliferation on micro-scale rough surfaces (Bačákova *et al.* 2001, Lossdorfer *et al.* 2004, Tan and Saltzman 2004), probably due to the fact that the cells were limited by the topography in their adhesion area.

However, the mechanism and the exact effect of microroughness still remain unclear. As several authors pointed out, this is especially due to the lack of a systematic study of this factor (Zhao *et al.* 2006, Stevens and George 2005). This problem can be directly related to the problem of defining roughness. In the previous studies the most widely used parameter Ra 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, Bacakova *et al.* 2004). 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.

2.3.7. Nanoroughness

While the effect of microroughness still remains controversial, considerable attention has been paid in recent years to the nanoscale structure of the material surface, which includes irregularities smaller than 100nm. Nanoarchitecture 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 have in mind that the natural environment of cells, the extracellular matrix (ECM), is also organized in nanodimensions (Fig. 5). In bone tissue it is composed mainly of the collagen type I fibers (with their diameter ranging between 10 and 300 nm) and of the inorganic component – hydroxyapatite nanocrystals about 4nm in size (Stevens and George 2005). Therefore many of the newly-developed bio-inspired composite materials try to mimic this effect of ECM on cells by constructing nanostructured surfaces. For example ifference between conventional and nanophase ceramics can be seen in Fig 6.

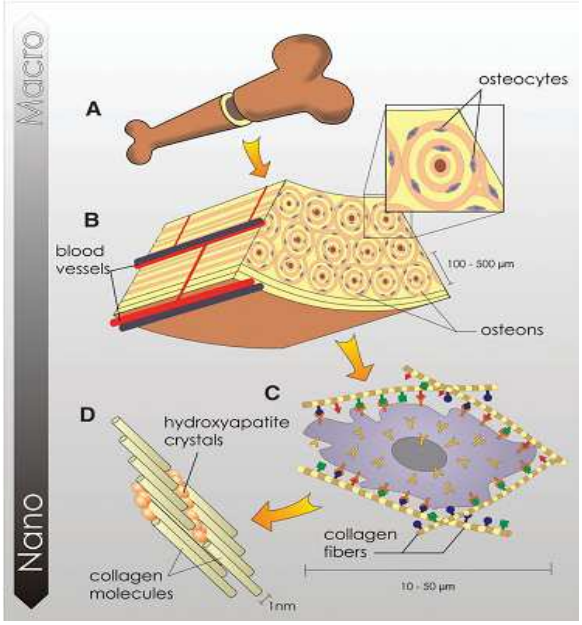
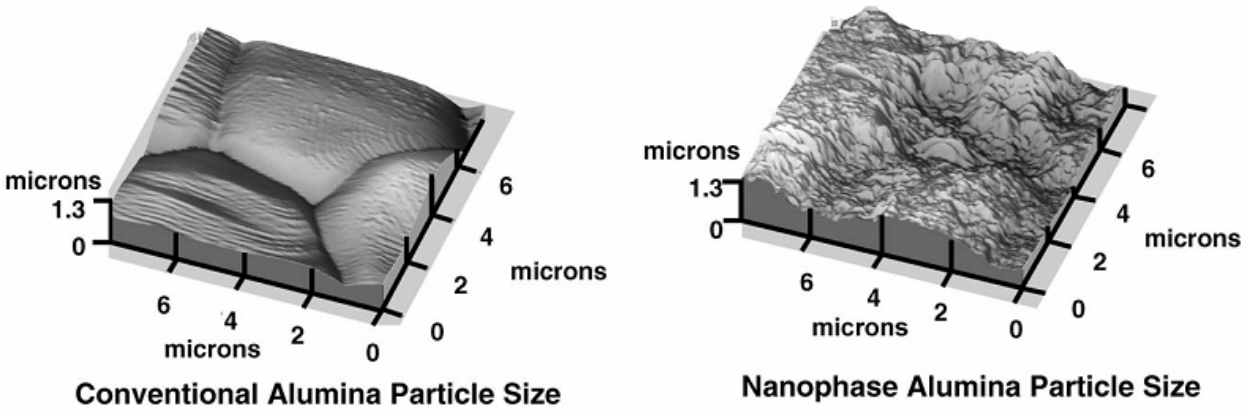


Fig. 5. Hierarchical organization of bone on different size scales, including nanoarchitecture of the extracellular matrix (reproduced from Stevens and George 2005).

Fig. 6. Atomic force micrographs of conventional and nanostructured ceramic material-alumina (reproduced from Webster and Eijofor 2004).



The mechanism of the nanoroughness effect has not yet been well elucidated. Several authors have reported increased protein adsorption, selective adsorption of certain protein types, as well as an improvement in the spatial conformation of the proteins adsorbed to nanostructured surfaces (Webster *et al.* 2000, Woo *et al.* 2003). For example, in a study performed on three dimensional poly-L-lactide scaffolds, Woo *et* al. (2003) observed that scaffolds with pores that had nanofiber architecture of their walls adsorbed four times more serum proteins than scaffolds with solid wall pores. What is more, the adsorption was selective showing enhancement for vitronectin and fibronectin, even though both types of scaffolds were made of the same material. The selective adsorption of vitronectin and osteoblast adhesion was attributed to the appropriate size and 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). The preferential adsorption of vitronectin to nanostructured surfaces has been attributed to preferential support of osteoblasts adhesion on these surfaces in comparison with other osteoblast-competitive cell types. While adhesion of bone cells is strongly stimulated by nanoroughness, the adhesion and cell number decreased significantly, especially in the case of fibroblasts, when compared with conventional surfaces (Price *et al*. 2004, Webster *et al*. 2000). Similarly, Kim *et al.* (2006) have observed that scaffolds in which pores had a nanoarchitecture (hydroxyapatite nanoparticles) showed bone formation after 8 weeks upon subcutaneous implantation into athymic mice, while in scaffolds with unmodified pores they were filled only with fibrous connective tissue (Kim *et al*. 2006). This selectivity could be highly advantageous, as it could help prevent the formation of fibrous tissue upon implantation - one the major problems of all currently-used materials - and thus lead to faster integration of the implant.

Webster *et al.* (2000) also speculated that the higher adsorption of ECM proteins to nanostructured surfaces in general could be the result of changed surface energetics and consequently of increased wettability of nanophase ceramics in comparison with the conventional types. Klabunde *et al*. (1996) have studied the surface chemistry of nanomaterials in greater detail, and they have proved that the nanostructure creates an increased surface area of the material, and thus changes the distribution of functional groups on the surface, as schematically shown, e.g., in Fig. 7 (Sato and Webster 2004). Higher exposure of these functional groups alters the surface wettability in comparison with conventional materials of the same chemical composition. This effect has been observed not only on ceramic materials, but also on polymers such as poly(glycolide-co-lactide) with embedded nanoparticles of alumina ceramics (Kay *et al.* 2002) as well as on metallic implants with nanotopography (Webster and Eijofor 2004).

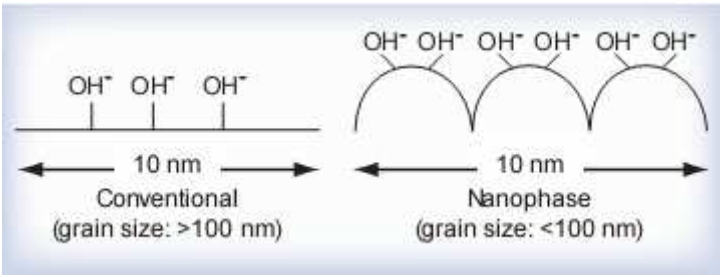


Fig. 7. Exposure of functional groups on the surface of a conventional and a nanostructured material surface.
From: Sato and Webster (2004).

Despite all these interesting findings, several studies have shown that for increased osteoblast adhesion in general nanostructure plays a bigger role than surface chemistry. This can be

illustrated especially by the experiment of Price *et al*. (2004), who compared the effect of carbon nanofibers with conventional carbon fibers (diameter 200nm), as well as the polyglycolide-lactide casts of both these samples. Interestingly, osteoblast adhesion was higher on nanofibers irrespective of the underlying material (Price *et al.* 2004).

Finally, not only the amount and type of the adsorbed proteins is changed on nanorough surfaces, but the bioactivitity of these proteins is also increased. It is believed that the nanotopography has a substantial influence on the conformation of the adsorbed proteins and the availability of specific cell binding domains (RGD sequences), because they are of the same size magnitude (Webster *et al*. 2000).

3. Polymer-based composites

3.1. Materials used or tested for bone implants

Currently used and tested materials designed for bone implants include especially metallic alloys, ceramics and synthetic polymers. All of these materials have certain advantageous properties, but all of them have been proven also to 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 titanium. Metals were chosen as the most 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. This stimulates remodeling of the surrounding tissue by bone 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.

Recently-tested materials include implants based on carbon, e.g. carbon composites with carbon or polymer matrix reinforced with carbon fibers, which were thought to be very promising due to their excellent mechanical properties as well as biocompatibility. However, there has been some concern about the fact that in some *in vivo* studies they have been shown to release small particles and debris (Bacakova *et al.* 2001, Lewandowska-Szumiel *et al.* 1999). A brief summary of advantages and disadvantages of these materials is given in Table 1.

Table 1. Materials for use in the body, and their advantageous/disadvantageous properties (reproduced and modified from Park and Bronzino 2003)

Materials	**Advantages**	**Disadvantages**
Polymers (nylon, silicone, rubber, polyester, polytetrafluoroethylene, etc.)	resilient, easy to fabricate	not strong, deform with time, may degrade
Metals (Ti and its alloys, Co-Cr alloys, stainless steel, Au, Pt, etc.)	strong, tough, ductile	may corrode, dense, difficult to make
Ceramics (aluminium oxide, calcium phosphates including hydroxyapatite, etc.)	very biocompatible, inert, strong in compression	brittle, not resilient, difficult to make
Carbon based-composites	strong, light, biocompatible	release of particles

3.2. Composite materials

Composite materials by definition are materials consisting of two or more different constituents at a micro- or macrosize range having a distinct interface separating them. Their major advantage is that they offer the possibility to combine properties of the initial material to engineer a new construct with 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 (Migliaresi 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). 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 and the inorganic particulate component – crystals of hydroxyapatite. 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.

In the following part of my thesis I will describe a novel polymer-based composite material that was constructed in a similar pattern as has just been described.

3.3. Composites based on polyamide reinforcement and polysiloxane matrix added with hydroxyapatite

The major advantage of polymers, as already mentioned, is their biocompatibility and relatively easy and cheap fabrication. Their insufficient mechanical properties can be improved by reinforcing them with metallic, ceramic or carbon fibres. However, these properties could also be markedly improved by reinforcing the polymer matrix with polymer fibres. These polymer-polymer composites, closely resembling the architecture of natural bone, seem to be promising in recent bone tissue engineering. Table 2. summarizes the mechanical properties, e.g., Young's modulus of elasticity and tensile strength of the natural bone and some of the polymeric materials used in medicine.

Table 2. Mechanical properties of biomedical polymers and bone (Reproduced and modified from Cooper *et al.* 2004, Wang 2003)

Polymer	**Bulk modulus (GPa)**	**Tensile strength (MPa)**	**Elongation at break (%)**
Polyethylene	0,8-2,2	30-40	130-150
Polypropylene	1,6-2,5	21-40	100-300
Polydimethyl-siloxane		3-10	50-800
Polyurethane	1,5-2	28-40	600-720
Polytetrafluoro-ethylene	1- 2	15-40	250-550
Polyamides	2,4-3,3	44-90	40-250
Polymethyl-methacrylate	3-4,8	38-80	2,5-6
Polycarbonate	2,8-4,6	56-75	8-130
Cortical bone	**7-30**	**5-150**	**1-3**

Therefore, for the construction of our newly developed composites, three materials were used: polymeric matrix made of polysiloxane, fiber-reinforcement from polyamides and hydroxyapatite. The combination of the first components should provide not only the necessary stiffness required for load-bearing implants, but also the required elasticity which would prevent bone resorption and implant loosening caused by the modulus mismatch (Wang 2003). However, the mechanical properties are not the only important factors: biological compatibility is of equal value. Therefore, the third component, hydroxyapatite particles, is included in the matrix, which should not only further enhance the mechanical properties but also render bioactivity to the construct.

3.3.1. Polyamide fiber reinforcement

Polyamides (or nylons) were chosen as the reinforcement material for this new composite, especially on account of their superior mechanical properties. They are known to be very light and strong with modulus going up to 190GPa (Migliaresi and Alexander 2004). Due to their interchain hydrogen bonding they also have very good fiber forming ability. Thanks to their long-time durability and resistance to mechanical stresses (especially pulling forces), they have been previously used for the construction of non-absorbable scaffolds of artificial joint discs (Springer *et al.* 2001). These studies have shown that the material is biocompatible and non-toxic, as it has supported growth of human fibrocartilage cells isolated from the temporomandibular joint in a comparable way to other polymers used in medicine, such as polyglycolic acid. In addition, polyamide fibres have already been used in constructing composites with nano-hydroxyapatite that matched very well the mechanical properties of natural bone (Wang *et al.* 2002). The authors emphasize the presence of carboxyl and amide groups in the polyamide chain similarly as in proteins like collagen, which are able to interact with the calcium atom in the molecule of hydroxyapatite. This interface linkage substantially influences the resulting mechanical properties of the material.

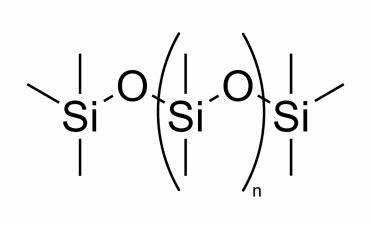
In terms of mechanical properties, aromatic polyamides (aramids) are of special interest in constructing the materials. Fibres made of these polymers have a specific strength five times that of steel, they have particularly high resistance to stress rupture, small elongation, low mass and they are able to resist abrasion damage (Migliaresi and Alexander 2004). Commercially available aramid known as Kevlar is used, for example, in bullet-proof vests. In biomedical application they have been in limited use for ligament prostheses, e.g., artificial tendons. However, they have not been extensively used in biomaterials, because there has been some concern about their biocompatibility and long-term fate (Migliaresi and Alexander 2004). These doubts were addressed by Zywicka

(2004) and her extensive biocompatibility study, which has shown that these fibres are highly suitable for medical applications. Aramid was proved not to cause any toxic, haemolytic or irritating action in the body in comparison with polyesters, which are already in extensive use in medicine. They did not cause increased levels of pro-inflammatory cytokines (IL-1beta and IL-6) after surgery. Furthermore, they were proved to be highly stable in the body, after 360 days of implantation into the muscles and into the peritoneal cavity of rats, no biocorrosion was observed. Moreover, their resistance to cyclic fatigue after this time was 200 times higher than that of polyesters, which proves their suitability for increasing the mechanical resistance of biomaterials.

3.3.2. Polysiloxane matrix

Reinforcing polyamide fibres will be molded in a matrix made of another polymeric material, polysiloxane (poly-**sil**icon-**ox**ygen-alk**ane**). These polymers differ, because their major chain contains silicone atoms. The general structure and mechanical properties are shown in the Fig. 8 and Table 2, respectively. Polydimethylsiloxane (PDMS) has previously been well studied in biomaterials science especially because of its high flexibility, which allows the formation of various microtextures on the material surface and it is easy to manufacture (Mata *et al.* 2002).

Fig. 8. Structure of polysiloxane.



The major disadvantage is its high hydrophobicity, with water angle about 110° (Liao *et al.* 2003), which significantly reduces cell adhesion and proliferation, as already mentioned above. However, the material is known to be biocompatible and it does not cause immune responses, macrophages cultured on PDMS produced lower levels of cytokines (IL-1beta, IL-6, TNF-alpha) than when cultured on polystyrene or polyethylene (Anderson *et al.* 1995). It is also able to support the growth of osteoblast-like cells, as shown by Gumula and Blazewicz (2004). The adverse effect of hydrophobicity of materials can be reversed by oxygen plasma treatment, which increases the level of hydrophilicity (Liao *et al.* 2003, Wei *et al.* 2007) or by the above-mentioned microtexturing of the surface (Liao *et al.* 2003, Mata *et al.* 2002).

3.3.3. Nanohydroxyapatite particles

The third component of the newly constructed composite will be hydroxyapatite (HAp). This ceramic material belongs to a larger 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 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).

In the construction of bio-inspired composite materials, nanoparticles of HAp similar to those in the extracellular matrix are used. This 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), especially in porous scaffolds that cannot yet be used in load-bearing implants.

In addition, nano-HAp has been proved to have positive effects on cell-biomaterial interactions (Webster *et al.* 2000). In several studies it has been successfully used to enhance the biocompatibility of a newly constructed material (Kong *et al.* 2005, Kim *et al.* 2006). For example, Kong (2005) described the preparation of a porous chitosan composite with nano-HAp particles (70-100nm wide, 140-260nm long) evenly dispersed on the pore wall surface. The osteoblast cells showed significantly higher proliferation and spreading on these scaffolds in comparison with scaffolds without HAp. Kim *et al*. (2006) developed a new method for preparing polymer (polyglyco-co-lactide) scaffolds with nano-HAp to ensure that the particles are exposed on the surface and not covered with the polymer. Scaffolds prepared by this novel method have shown higher cell numbers and a higher level of differentiation and mineralization than cells grown on scaffolds prepared by the conventional method, where most of the HAp particles are covered by the polymer matrix. Therefore, increased bone formation can be directly related to the contact of osteoblasts with nano-HAp particles. Balasundaram *et al*. have even suggested in their study that the positive effect of decreasing the size of HAp particles to nanoscale on osteoblasts 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.

By including nano-HAp particles in our newly-developed composite, we also hope to improve not only the mechanical properties of the composite, but also its biocompatibility, by exposing the HAp particles on the surface. This should as a result increase the cell proliferation and

differentiation and thus help to improve the non-favorable properties of the siloxane matrix for cell growth given by its inherent hydrophobicity.

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*. (2004) 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 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.

3.3.4. Porosity

Biomaterials can be constructed in two ways. Firstly, as implants with a simple two-dimensional interface where cells adhere only to the solid surface of the material. A more advanced, tissue engineering-like approach involves constructing porous materials with a scaffold architecture which should guide migration and ingrowth of cells inside the material, where they interact with the material in a 3D environment. This kind of strategy has been shown to elicit favorable and desirable responses. After the cells penetrate into the scaffold, they form new bone tissue inside the pores and thus they are able to anchor the implant in a much better way. The interlocking between the artificial material and the natural bone is stronger and more stable, thus preventing loosening of the implant with time. This strategy has also been applied to functionalize currently-used metallic implants by coating them with various porous layers (Karageorgiou and Kaplan 2005). Therefore, in the construction of this new composite material we will also introduce pores into its structure to enhance its integration into the natural bone tissue.

Many studies have been conducted which tried to establish the optimal degree of porosity and especially pore size to maximize bone ingrowth. The results are quite different for different materials, cell types and in vitro vs. in vivo conditions. However, certain general trends are observable. Concerning porosity, the higher the percentage, the greater cell proliferation was observed, probably due to the increased space and surface area. The upper limit is in general set by the worsening mechanical properties, especially decreasing strength and higher brittleness as the porosity level rises.

Concerning pore size, the minimal pore diameter to allow penetration of bone cells is about 80 to 100μm (Karageorgiou and Kaplan, 2005). As was shown in a recent study by Pamula *et al*.

(2007), scaffolds with smaller pores (40μm) do not allow cell ingrowth, because the bone-derived cells are large enough to span the pores and form a continuous layer on the surface instead. Not only does small pore size limit the physical penetration of cells, but the cells can also suffer from insufficient nutrient exchange and hypoxia (Pamula *et al*. 2007). This study also showed in accordance with similar experiments that the optimal pore size was about 600μm.

In several experiments also reviewed by Karageorgiou it has been shown that in vivo larger pore size stimulated direct bone ingrowth. Pore diameter larger than 300-400μm was critical for neovascularization to occur, which consequently improved the oxygen and nutrient supply and thus stimulated ossification. In contrast, in pores of smaller diameter, osteochondral ossification occurred as the result of hypoxia (Karageorgiou and Kaplan 2005).

4. Experimental Part

In the experimental part of this thesis we have tested the biocompatibility of both polyamide reinforcing fibres and of the first constructed composites *in vitro* by evaluating the adhesion, growth and viability of human osteoblast-like MG 63 cells cultured on these materials.

All of the tested materials were prepared and characterized by Karel Balik, Zbynek Sucharda and Tomas Suchy from the Institute of Rock Structure and Mechanics, Academy of Sciences of the Czech Republic, and by Miroslav Sochor from the Czech Technical University in Prague, Faculty of Mechanical Engineering.

4.1. Materials and Methods

Polyamide, tested in the first stage, was in the form of commercially available woven fabrics made of aromatic polyamide fibres - Aramid plain weave cloth 20796 (Aramid Balanced Fabric, Hexcel Company, France) made of HM215 fibers with specific weight 1.44 g/cm^3, fiber diameter 13.5μm.

In the second phase, six different composite materials using the Aramid polyamide fabric as a reinforcement were constructed. Each composite sample was prepared with polymethylsiloxane matrix M130 (Lucebni Zavody Kolin, Czech Republic). Phosphoric acid was used as a copolycondensation catalyst. Two kinds of hydroxyapatite (HAp) were added as particle additives: HAp with particle size 10-100μm in diameter (LASAK Ltd., Czech Republic) and HAp with particle size of 10-40μm in diameter (School of Mechanical Engineering, Singapore).

The composite samples included:

A. Pure Composite – Aramid + siloxane 50%:50%

B. Comp + PO_4^{3-} – Aramid + siloxane + phosporous anion 50%:49,5%:0,5%

C. Comp + *pores* – Aramid + siloxane + pores 55,6% : 44,4%

D. Comp + *HAp 100* – Aramid + siloxane + hydroxyapatite (10-100μm) 40%:34%:9%

E. Comp + *HAp 100* – Aramid + siloxane + hydroxyapatite (10-100μm) 60%:26%:5%

F. Comp. + *HAp 40* – Aramid + siloxane + hydroxyapatite (10-40μm) 42%:40%:7%

A standard tissue culture polystyrene dish (TCPS) was used as the control sample.

4.1.1. Preparation of the material for the cultivation of cells

The Aramid weave cloth was cut into circles 2.1cm in diameter. The pressed discs of the composites were cut into samples 10x14mm in size. These were sterilized in an autoclave for 1 hour at temperature 120^{o}C and pressure of 1atm.

4.1.2. Cells and culture conditions

The samples were inserted on the bottom of polystyrene culture dishes (TPP, Switzerland, 12 wells of diameter 2.2 cm) and were seeded with human osteoblast-like cells of the MG 63 line (European Collection of Cell Cultures, Salisbury, UK) at an initial density of 21 000 cells/cm^2 in 3 ml of Dulbecco-modified Eagle Minimum Essential Medium with 10% of fetal bovine serum and 40 µg/ml of gentamicin. The cells were cultured at 37°C in a humidified atmosphere containing 5% of CO_2 for 1, 3 and 7 days.

4.1.3. Cell adhesion and growth

The cells were rinsed by phosphate buffer saline (PBS) and detached from the material by a trypsin - EDTA solution (Sigma, U.S.A, cat. N^{o} T4174; 5 min, 37^{o}C). The number of cells and their viability was measured using a ViCell XR analyzer (Beckman Coulter, U.S.A). From the number of cells we constructed the growth curves and calculated the doubling time according to the formula:

$DT=(t-t_o)\log 2/\log Nt-\log Nt_o$, where t_o and t represented earlier and later time intervals after seeding respectively, Nt_o and Nt the number of cells at these intervals (i.e., day 1, 3 or 7 after seeding).

4.1.4. Cell morphology

In order to evaluate the cell morphology, the samples were rinsed with PBS, fixed in 70% ethanol, stained with propidium iodide (5 µg/ml solution in PBS) on day 1 after seeding or Hoechst 33342 (Sigma, USA, cat.N^{o} B-2261; dilution 1:200) on the 3rd and 7th day of cultivation and then observed using an Olympus IX 50 inverted microscope, equipped with a DP 70 digital camera. The cells were also stained immunocytochemically against the cytoskeletal protein beta-actin (Bačáková *et al.* 2002). Briefly, as the primary antibody, monoclonal anti-β-actin (clone AC-15, Sigma, USA, cat.N^{o} A-5441) was applied in dilution 1:500 at 4^{o} C overnight. As the secondary antibody, an F(ab')2 fragment of Goat Anti-Mouse IgG (H+L) conjugated with Alexa Fluor® 488 (Molecular Probes, USA, cat.N^{o} A11017; dilution 1:500) was used for 1 hour at room temperature. Cells were then

observed using a using inverted microscope Olympus IX 50, equipped with a digital camera DP 70 or confocal microscope (Leica, TCS SP2, Germany).

4.1.5. Statistics

The quantitative data was presented as averages ± S.E.M. (Standard Error of Mean) from 3 samples. The statistical significance of the differences was evaluated by Student's t test for unpaired data or by one-way ANOVA, Student-Newman-Keuls Method (SigmaStat, USA).

4.2. Results and Discussion

4.2.1. MG-63 culture on Aramid fabrics

The Aramid weave cloth allowed cell adhesion and proliferation. However, the cell number on this material was significantly lower on all of the tested days than on the control polystyrene dish (Fig. 9). The cells also proliferated much more slowly, as indicated by more than two times higher doubling time between days 1 and 7 after seeding. However, it seemed that the lower colonization of the Aramid fabric was due to its irregular surface topography and its relatively high roughness, which hampered cell attachment and growth, rather than the cytotoxicity of the material. The viability of cells on the material was relatively high, more than 85%, which was comparable with the control sample. These results correlate with the observations of other studies that have proved the biocompatibility and non-toxicity of this polymer (Zywiczka 2004, Springer *et al.* 2001). Moreover, the cell morphology shown in Fig. 10 also indicates the good condition of the cells. The cells were well spread and attached to the material, although they were spindle-shaped and elongated in the direction of the underlying fibers, while on polystyrene they were rather polygonal. A similar parallel arrangement of elongated cells has been reported for a wide range of materials, for example on surfaces patterned by microgrooves (Tan and Saltzman 2004), and is a sign of the sensitive reactivity of MG 63 cells to the surface topography, sometimes referred to as contact guidance.

However, in the later days of cultivation the cells on the fabrics were able to cross the fibers and bridge most of the holes and grooves in the structure of the material. On the 7^{th} day of cultivation they already formed a confluent layer (Fig. 11).

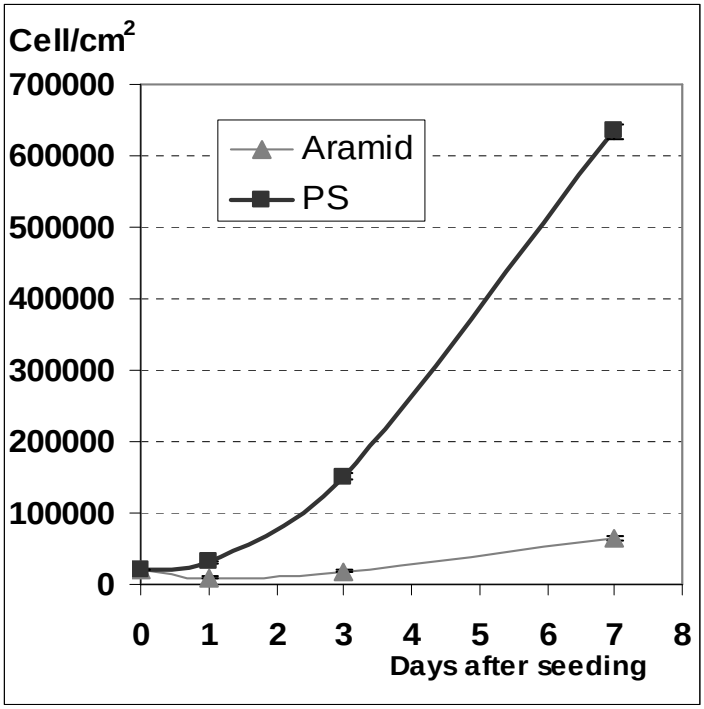
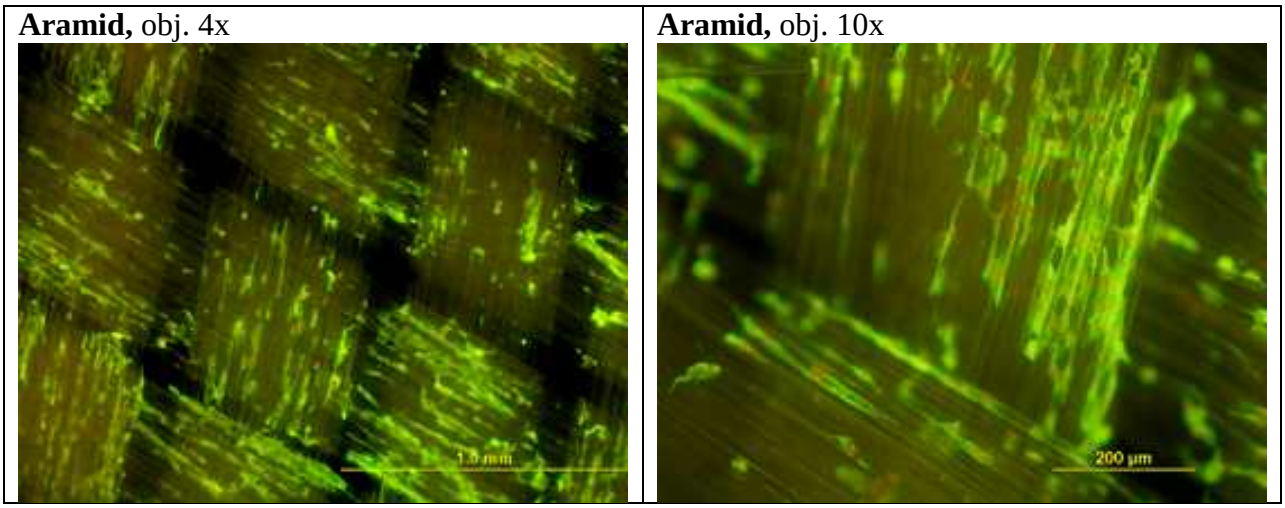


Fig. 9. Growth curve of viable human osteoblast-like MG 63 cells on Aramid fabrics and tissue culture polystyrene (TCPS).

Fig. 10. Morphology of human osteoblast-like MG 63 cells on Aramid fabrics, and tissue culture polystyrene (TCPS) on the 3rd day after seeding. Immunofluorescence staining of beta-actin; the nuclei were counterstained with propidium iodide. Olympus epifluorescence microscope IX 50, digital camera DP 70, objectives 4x and 10x.



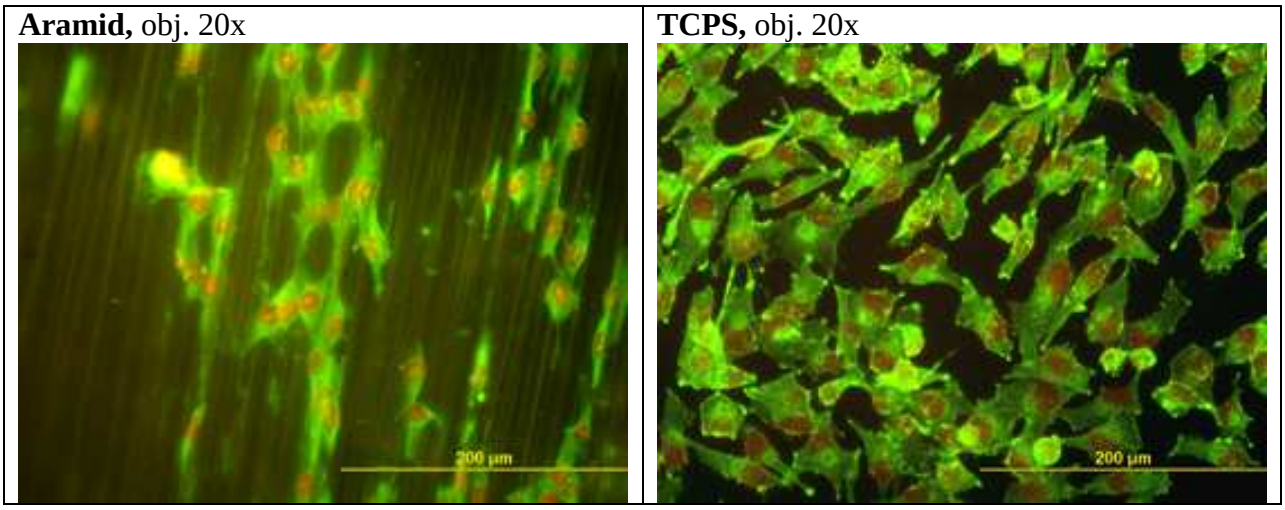
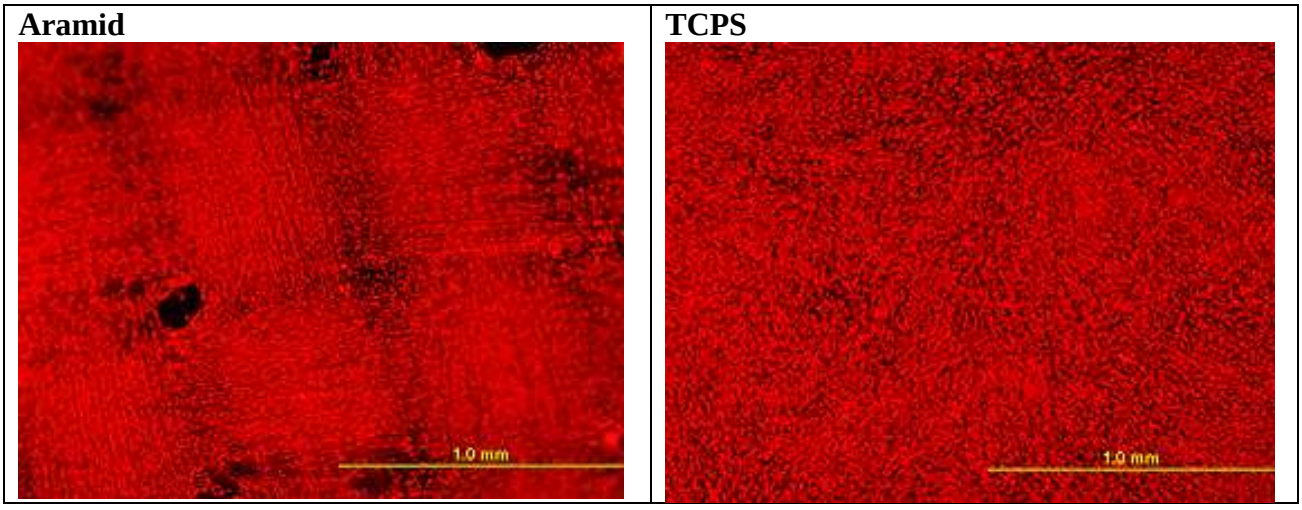


Fig. 11. Morphology of human osteoblast-like MG 63 cells on Aramid fabrics and TCPS on the 7th day after seeding. Stained with propidium iodide; Olympus epifluorescence microscope IX 50, digital camera DP 70, obj. 4x



4.2.2. MG-63 culture on Aramid composites

The Aramid composites allowed cell adhesion and proliferation, but similarly as in the case of the pure weave cloth, the cell number was always significantly lower than on the control polystyrene dish, probably due to the hydrophobicity of the siloxane matrix, which is known to be unsupportive for cell proliferation (Liao *et al.* 2003) and the high roughness of the material surface. In order to evaluate the effects of HAp particle inclusions on cell colonization, the results were compared with the basic composite, sample A. On the first day after seeding, the cell number was

comparable on all of the composites. Sample D-F, which contained HAp particles, tended to have a lower average number of cells, though this difference was significant only in the case of sample D. However, already on the third day of cultivation, the number of cells on the samples with HAp (D-F) showed no significant difference in comparison with A. On the other hand, on samples with phosphorous anion (B) and with pores (C) the number of cells still remained significantly lower than on the basic composite A. On composites B and C, a similar trend was continuously observable also on the 7th day of cultivation. The number of cells was significantly lower on all samples in comparison with composite A, but samples D-F reached 60-70% of sample A, whereas B and C reached only about 40%. Moreover, the cells on composites B and C showed a relatively low viability of 75% and 85%, respectively, whereas on the remaining samples, this value reached more than 90% on day 7 after seeding (Fig. 12). These results also correlated with a significantly longer doubling time of cells, which means slower proliferation, on samples B and C, whereas on the other samples it was comparable with sample A and even with the control polystyrene (Fig. 13).

Fig. 12. Number of human osteoblast-like MG 63 cells on the 1st, 3rd and 7th day of cultivation on the tissue culture polystyrene (TCPS) and the composite materials of the groups labeled as A-F (see Material and Methods) and the viability of cells on the 7th day. Means ± SEM. ANOVA, statistical significance: $^{A, B, C}$: $p \leq 0.05$ in comparison with the materials of the groups A, B, C.

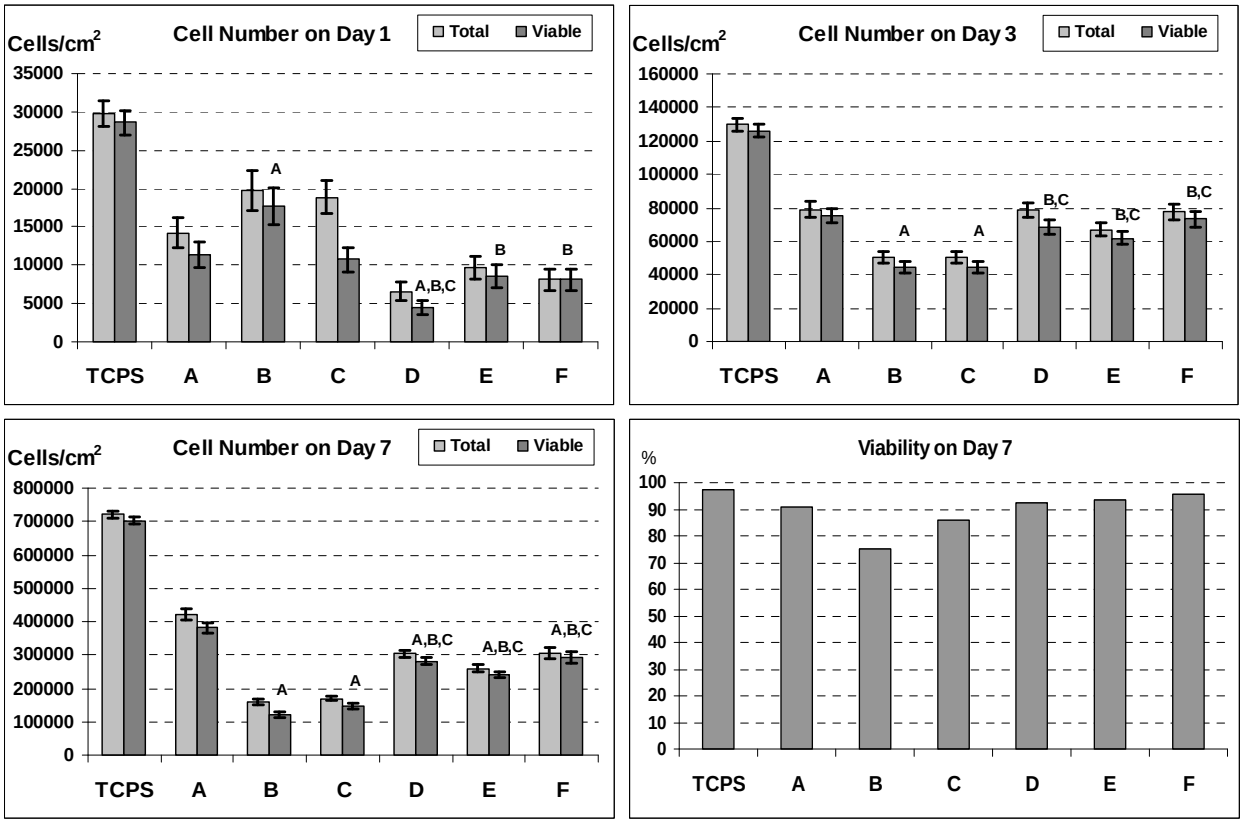
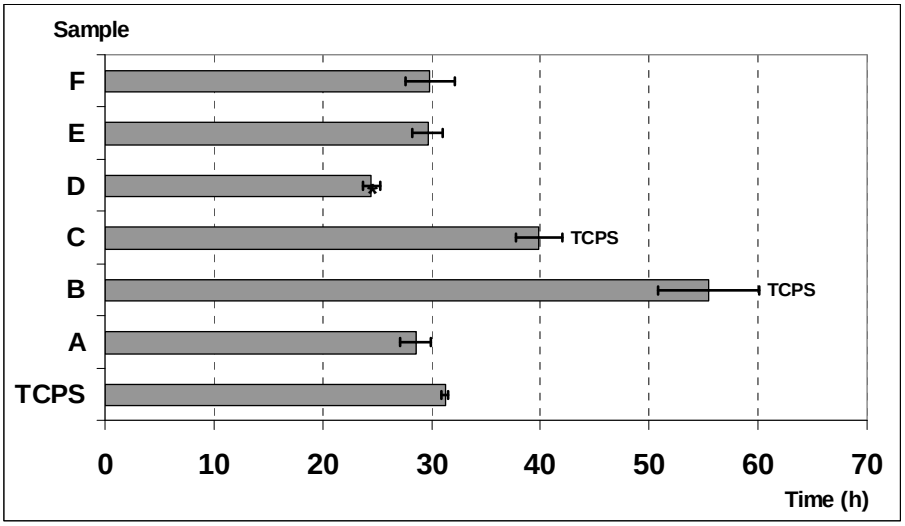


Fig. 13. Doubling time of human osteoblast-like MG 63 cells on the tissue culture polystyrene (TCPS) and the composite materials of the groups labeled as A-F (see Material and Methods) between days 1 and 7 after seeding. Means ± SEM. ANOVA, statistical significance: ^{TCPS}: $p \leq 0.05$ in comparison with the values on tissue culture polystyrene.



The cell morphology (Fig. 14) again indicated good vitality of the osteoblast-like cells adhering to the composites. As revealed by the immunofluorescence staining of beta-actin, the cells were polygonal in shape and well spread. Some of them were spindle-shaped and elongated in the direction of the reinforcing fibers. Already on the 3rd day after seeding, a confluent layer began to form on the surface of the composites, similarly as on the polystyrene dish. On the 7th day the cells completely covered the surface of all the samples, and there was no observable difference in comparison with the control (Fig. 15).

Fig. 14. Morphology of human osteoblast-like MG 63 cells on composite materials (see Materials and Methods) and control tissue culture polystyrene (TCPS) on day 3 after seeding. Immunofluorescence staining of beta-actin; the nuclei were counterstained with propidium iodide. Confocal microscope Leica TCS SP2 (Germany), obj 10.

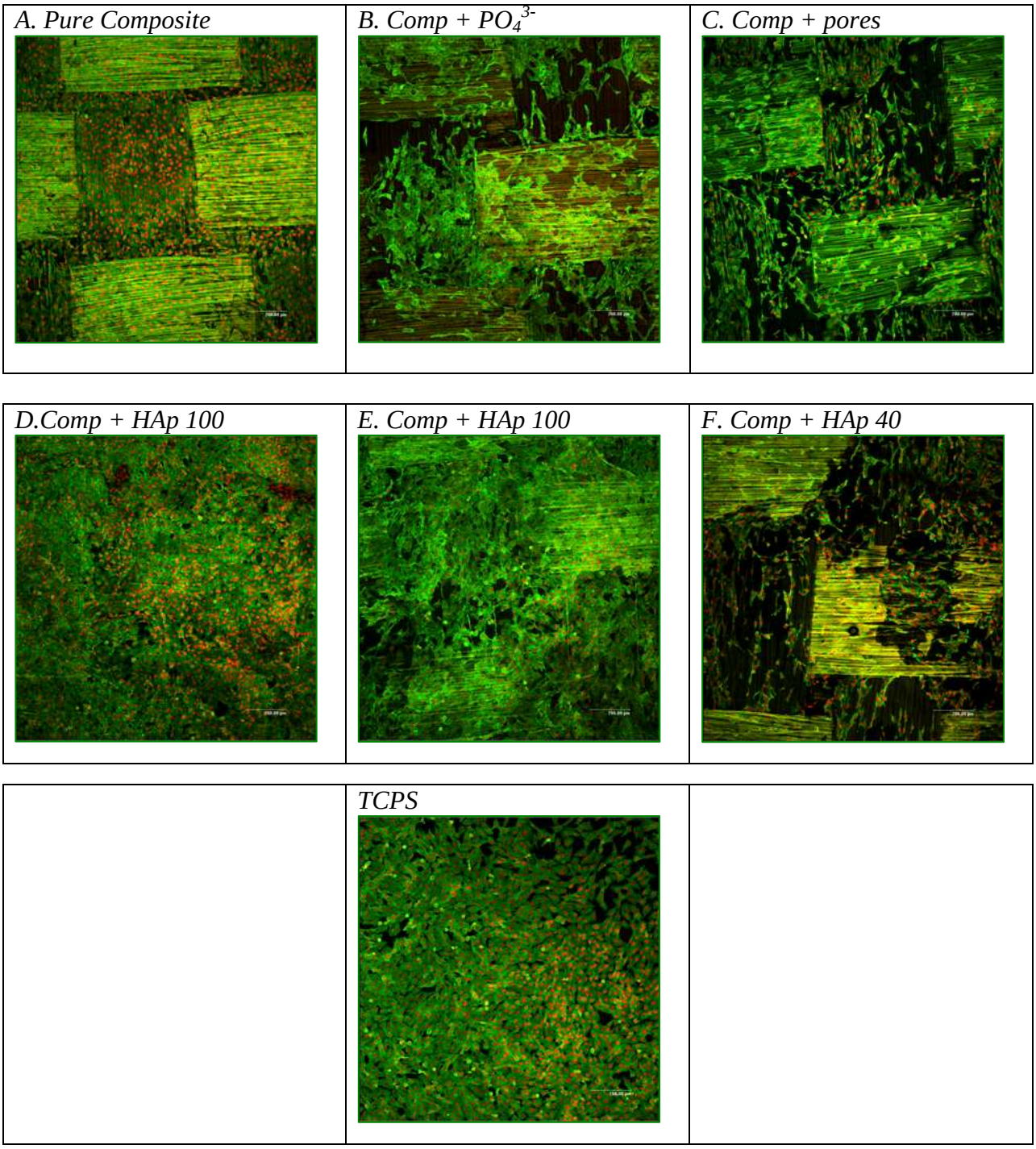
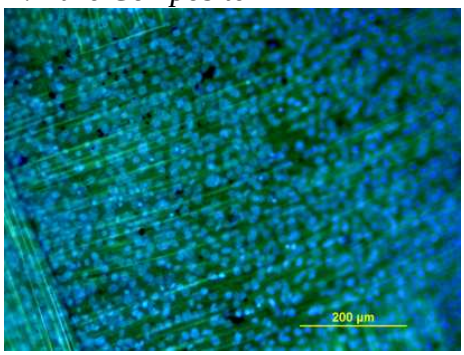
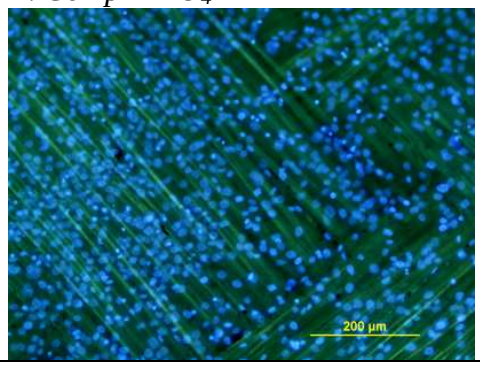


Fig. 15. Morphology of human osteoblast-like MG 63 cells on composite materials (see Materials and Methods) and control tissue culture polystyrene (TCPS) on day 7 after seeding. Stained with a nuclear stain Hoechst 33342; Olympus epifluorescence microscope IX 50, digital camera DP 70, obj. 10x

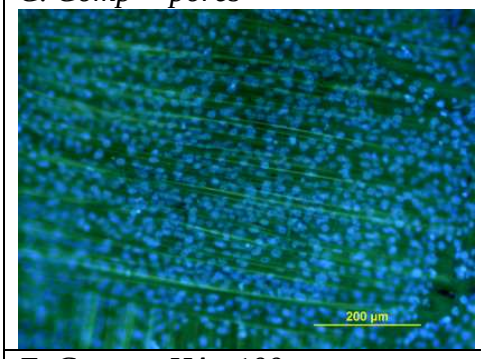
A. Pure Composite



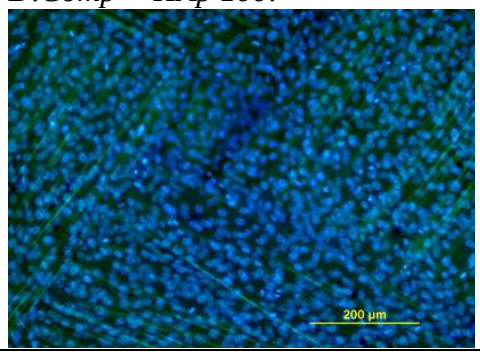
B. Comp + PO_4^{3-}



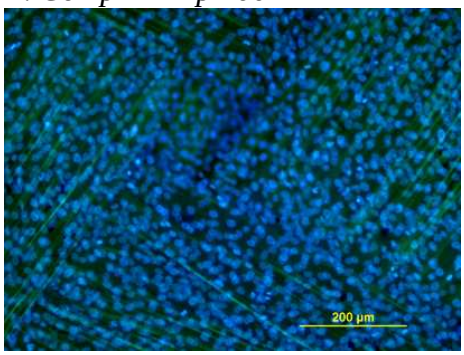
C. Comp + pores



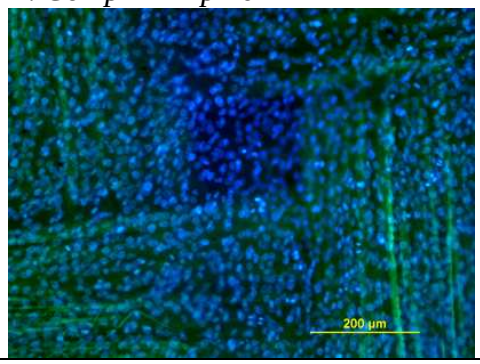
D.Comp + HAp 100.



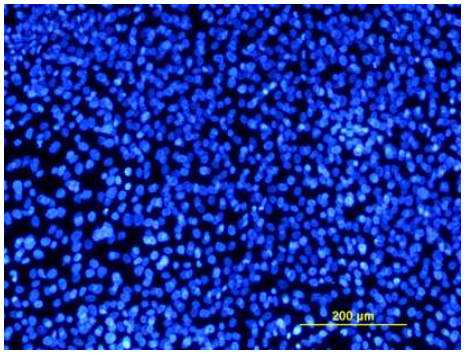
E. Comp + HAp 100



F. Comp + HAp 40



TCPS



4.3. Conclusions and future research

The results obtained in this study lead to the conclusion that the newly developed composites are promising for use in hard tissue surgery after several improvements and modifications. These changes should lead to further functionalization of the material surface, which has the most important role in cell-biomaterial interaction. Modifications could possibly include smoothing the sample surface in order to make it more suitable for cell spreading. Microscale roughness as the result of the structure of reinforcing fibers (diameter 13,5μm) was proved to have an influence on cell adhesion and spreading, as shown by their morphology partially following the organization of the fibers, though these were embedded in the matrix. Furthermore, the HAp particle inclusions could have increased the roughness as their size was very variable, but generally it also ranged in micrometers (100-10μm or 40-10μm). As mentioned above, the effect of microroughness on cell response still remains controversial, and was even shown to slow down cell proliferation (Bačákova *et al.* 2001, Tan and Saltzman 2004). Chou *et al.* (2004), who examined the effect of different types of HAp particles on cell response, also reported lower cell numbers on samples with micro-HAp particles. In combination with the hydrophobicity of the siloxane matrix, this could be the most probable explanation of lower colonization of our newly-developed materials by MG 63 cells. This colonization could be improved, e.g., by polishing the material surface, optimally to nanoroughness, together with increasing its wettability by coating it with a smooth biocompatible layer.

Other major modifications could therefore include decreasing the size of the HAp crystals strictly to nanoscale (smaller than 100nm), which would more closely mimic the natural inorganic component of bone tissue. As mentioned above, enhanced adhesion and proliferation of osteoblasts has been related directly to nanostructure in general, including nanosize HAp particles (Kim *et al.* 2006). For the construction of future composite materials, testing of several different types of HAp particles would be advisable, as not only the size but also the shape and crystallinity of the particles have been shown to play a crucial role (Chou *et al.* 2004).

In future, a three-dimensional porous design of the material, which would enable its inner colonization with cells, should be carefully elaborated. In our porous sample (sample C), the pores were created rather only on the surface, which distorted its smoothness even more, and thus had an adverse effect on cell numbers, as shown by our results.

However, if these modifications were to further enhance the biocompatibility of these newly developed composite materials, they would be promising for future use in clinical practice, for example for constructing bone and joint replacements, intervertebral discs, external and internal bone fixations, or for the use in dental and maxillofacial surgery.

5. General conclusion

Composite materials are a strategy for constructing biomaterials that will meet the specific requirements on mechanical properties imposed by the type of tissues that they are to replace. However, in the past two decades, increasing emphasis has also been laid on the bioactivity of the materials designed for implantation into the body.

To be able to reach this goal, the mechanism of cell adhesion and interaction with an artificial material must be well understood. Some of the basic principles of protein adsorption and subsequent receptor-mediated cell binding to the material surface have already been elucidated. However, there are still certain areas that await deeper investigation, e.g., cell adhesion mediated by non-integrin receptors, which has been particularly shown to be influential and important in osteoblast adhesion to bone implants (Dee *et al.* 1998).

Strategies that aim at improving the biocompatibility and bioactivity of the material include especially modifications of the surface physico-chemical properties, e.g., surface chemistry, wettability and topography. These factors and their influence on cell response have been of particular interest to many biomaterial research groups. The results and details of the underlying mechanisms however remain in many cases incomplete (e.g. microroughness). Furthermore, as these factors influence cell response concurrently in a complex way, it is often quite difficult to interpret the results. Therefore, biologists are still not able to give material engineers an unequivocal answer about the properties that the material should possess in order to elicit the desired response and integration into the natural tissue.

Since approximately 2000, significant attention has been paid to nanotechnologies. It has namely been shown that nanostructures could be advantageously used also in the field of biomaterials and especially in bone tissue engineering. Materials with nanostructured surfaces have been observed to enhance osteoblast cell adhesion, proliferation and differentiation. Moreover this enhancement seems to be selective for bone cells, which could give the material very promising and advantageous properties. The mechanism of increased cell adhesion has been shown to lead to higher and selective protein adsorption, however the explanation of this phenomenon still remains rather a matter of speculation and is currently being investigated. Interestingly, the positive effect of nanoroughness on cells seems to be independent of the surface chemistry of the material (Sato and Webster 2004). Furthermore, the enhancing effect of the nanostructure seems to be really powerful and comparable with other highly advanced strategies, such as incorporating synthetic proteins with specific integrin-binding domains (RGD sequences) onto the material surface (Balasundaram *et al.* 2006). If this observation proves in future to be a general principle, introducing nanostructure could

become a powerful tool for improving and increasing the functionality of various types of bone implants. On the other hand, 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.

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