Nanostructured Materials for Skeletal Repair

Joerg Brandt,[*1] *Goerg Michler,*[2] *Juergen Vogel,*[3] *Sven Henning*[2]

Summary: The treatment of bone and cartilage defects with bioengineered constructs of artificial scaffolds and autogenous cells became the main challenge of contemporary regenerative medicine. Early defect repair may prevent secondary injury. Recent studies could prove that bone and cartilage cells are sensitive to microscale and nanoscale patterns of surface topography and chemical structure. Nanostructured materials provide an environment for tissue regeneration mimicking the physiological range of extracellular matrix. The article reviews several studies substantiating the superiority of nanostructured materials for bone and cartilage repair along with own results on cell attachment.

Keywords: biomaterials; nanofibres; tissue engineering scaffolds

Introduction

Traumatic lesions as well as tumor derived defects in bone and cartilage may generate severe consequences for function and longevity of skeletal system and joint function. Small substance defects can cause severe secondary dysfunctions and limits in the range of motion, working capacity and the quality of life. Skeletal and osteochondral lesions are frequent injuries of younger aged patients after sports injuries and traffic accidents and may cause lifelong restriction due to degenerative diseases with secondary onset. With the development of sophisticated cell culture techniques a new discipline was established in the clinical practice: the regenerative medicine. Several surgical procedures are in clinical use to restore traumatic damage of bone and hyaline cartilage including autogenous and allogenous tissue grafts. The clinical availability of autografts is restricted by problems like the concomitant morbidity of the surgical approach and the limited amount of donor tissues. The disadvantages associated with allografts are the immunological problems of incompatibility or tissue rejection and the pathogen transmission. In case of bone and cartilage besides these general transplantation-derived problems a vital transplant with functioning blood circulation is rather achievable. Since the nineteen-seventies great efforts have been undertaken to find appropriate artificial substitution for bone and cartilage. The modification of traditional surgical materials at a macroscopic level could not meet the demands to reconstruct specific nanoscale structures.[1] The nanoscale structure of the extracellular matrix basically influences the spreading and behavior of cells forming a sophisticated network of fibrils and fibers. It promotes and regulates physiological cell functions such as proliferation, differentiation, migration and morphogenesis. The cells respond to the matrix by means of plasma membrane receptors requiring the coordination of a multiplicity of signals.[2–4] The rapid development of materials science created the basic technologies to engineer nanoscaled materials mimicking the dimensions of physiologic extracellular environment.[5–7] This review

[1] Orthopedic department, Martin Luther University Halle-Wittenberg, D-06097 Halle, Germany
Fax: (+49) 345 557 2306;
E-mail: joerg.brandt@medizin.uni-halle.de
[2] Institute of Physics, Martin Luther University Halle-Wittenberg, von-Danckelmann-Platz 3, D-06120 Halle, Germany
[3] Institute of Physics, Martin-Luther-University Halle-Wittenberg, Heinrich-Damerow-Str. 4, 06120 Halle, Germany

is aimed to summarize these material developments for the substitution of skeletal defects.

Scaffold Materials for Cartilage Tissue Engineering

Since Brittberg[8] first described the isolation and cultivation of chondrocytes from human cartilage a rapid development led to tissue-engineered cartilage as in vivo substitutes, produced by expansion of living chondrocytes. After separation from the extracellular matrix the cartilage cells (chondrocytes) dedifferentiate, change their outer shape and lose their cartilage-specific functions (as the production of glucosaminoglycans, typical proteins providing the immense water-binding capacity of cartilage) but they start cell division and proliferation. By means of adequate culture media billions of cells thus can be gained in a couple of weeks. Brittberg used the amplified cells to repair cartilage defects in young adults caused by traumatic injury of greater extremity joints. During this procedure of autologous chondrocyte transplantation (ACT) all connective tissue is surgically removed from the defect and the walls are smoothed. Than the defect can be covered with periosteal flap and sealed impermeably with fibrin glue. Finally the in vitro amplified chondrocytes can be replanted into the preformed cavity. Since the introduction of the method in 1987 full-thickness cartilage defects in more than 12,000 patients had been treated worldwide until 2006.[9] Even though the defect was bridged and good clinical results were reported in isolated post-traumatic lesions of the knee joint in younger patients[10] later histological investigations revealed that the regenerate does not resemble normal hyaline (= articular) cartilage but quarried a fibrocartilage regenerate with huge amount of cells and only small percentage of extracellular matrix. The method could therefore not fully restore a physiologic joint surface and it was put into question if the regenerate is able to improve the long-term survival of the joint.[11] A randomized, blinded study in patients with articular defects in the knee joint could find no significant difference between ACI and the simple surgical procedure of microfracturing (i.e. fracturing the subchondral bone layer at the bottom of the chartilage defect) two and five years after surgery.[12]

Articular cartilage provides a smooth, near frictionless surface which is not reached by any synthetic gliding combination yet. The thin layer of hyaline cartilage dissipates the load from the joint surface and transfers it to the subchondral bone plate. The unique microarchitecture of extracellular cartilage matrix facilitates the load transfer and provides resistance to tensile, compressive, and shear stresses. Unlike other tissues including fibrocartilage in the hyaline cartilage the major part of the volume, about 85% consist of extracellular matrix and only 15% are taken by chondrocytes. It means that the extracelluar matrix overtakes the biomechanical function of the cartilage and the small number of cells only responsible for its preservation and regeneration. The matrix contains a large amount of different proteins for maintaining interior pressure and lubrication, promotes cellular adhesion, proliferation and differentiation.

Since the clinical studies of long term results after autologous chondrocyte transplantation (ACT) of a simple cell suspension according to the procedure described by Brittberg[8] revealed a fibrocartilage regenerate with randomly ordered cell clusters and not the restoration of physiologic articular cartilage,[13,14] intense efforts had been made to create three dimensional implants containing an adequate amount of cells and leaving space for the development of ECM. An ideal scaffold for cell replantation should fully disappear during the growth of ECM molecules without leaving remnants. Cell culture experiments could show that a 3D environment of the ECM effects cell shape and the differentiation process by communicating both spatial and temporal information to adherent cells.[15,16] The figure below illustrates the fundamental change in morphology of

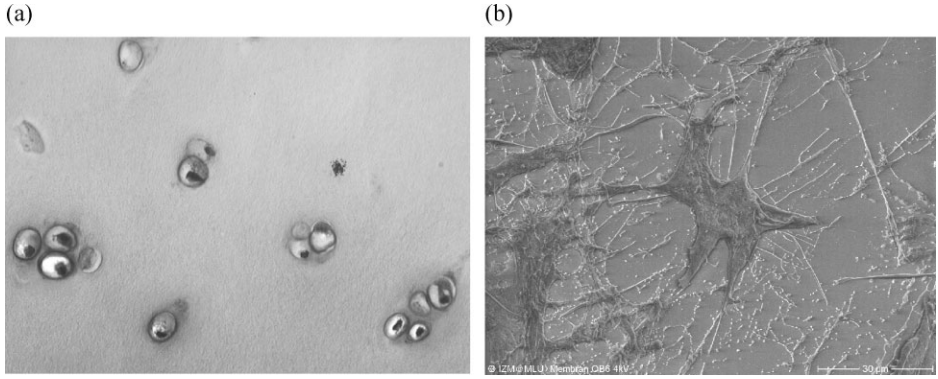

Figure 1.
a) shape of chondrocytes in normal hyaline cartilage; b) chondrocytes in 2D culture.

chondrocytes in 2D culture and 3D culture (Figure 1).

To overcome the disadvantages of ACT a lot of trials had been undertaken to find appropriate scaffolds for replantation of the cell into the defect. The first generation of scaffolds consists of solid, porous polymers for cell seeding. Gugala et al [17] used three-dimensional porous scaffold from poly(L/DL-lactide) 80/20% to culture Sheep articular chondrocytes. The chondrocytes on the polylactide scaffold maintained their round shape and the authors described a proceeding cell ingrowth into the sponge with time of culture. At 9 weeks, the chondrocytes filled the whole scaffold and reached the opposite side of the sponge. Cell growth and activity was estimated from the amount of proteoglycans attached to the polylactide scaffold and the amounts of DNA increased with study time indicating increasing cell numbers.

The combination of poly(DL-lactic-co-glycolic acid) (PLGA) with collagen microsponges in the pores of PLGA sponge was used by Chen et al to form biodegradable hybrid scaffolds of synthetic and natural polymers.[18] In vitro studies with bovine articular cells showed that hybridization with collagen facilitated cell seeding in the sponge and raised seeding efficiency. Chondrocytes adhered to the collagen microsponges, proliferated and produced extracellular matrix filling the space within the sponge. The authors reported a more homogeneous tissue formation in hybrid sponge than in PLGA sponge after subcutaneous implantation in nude mice.

The search for new scaffolds focused on biodegradable polymers that have been FDA approved for use in humans: poly-(glycolic acid) (PGA), poly(L-lactic acid) (PLLA) and their copolymer, poly(DL-lactic-co-glycolic acid) (PLGA).[19–22] Due to their common chemical nature poly(a-hydroxyesters) allow a degradation by simple hydrolysis. The more hydrophobic and less crystalline PLLA degrades at slower velocities in body fluids than PGA.[23]

In animal experiments hyaline-like cartilage formation has been observed six weeks after seeding undifferentiated perichondrial cells onto PLLA meshes and implantation in the distal femur of rabbits by Chu et al.[24] Comparable results were reported from in vitro experiments with bovine chondrocytes on PGA scaffolds. Twelve weeks after seeding mechanical properties as compressive modulus reached the value of normal bovine cartilage.[25] The proliferation of cells seems to depend on substrate properties: initial proliferation rates of bovine chondrocytes were approximately twice at high on PGA compared to PLLA possibly due to slower degradation of the material leaving less space for invasion of new cells. Interestingly after

long-tem cultivation of six months the total cell numbers reached equal amounts.[26]

The first clinically relevant results with natural derived polymer materials had been reported by Cherubino et al. [13] In 13 patients with deep cartilage defects of the knee joint they filled the lesion with autologous chondrocytes cultured in a type I/III bilayer membrane of collagen. The procedure was named matrix-induced chondrocyte implantation (MACI) and spread quickly due to its easier handling and surgical procedure compared with ACT. Electron microscopy of the precultured matrix revealed single standing chondrocytes producing sparse collagene fibers. Six months following replantation magnetic resonance imaging (MRI) showed signals similar to hyaline cartilage.

Hyaluronic acid based material (Hyalograft C) had been reported to deliver good clinical results in knee cartilage defects by Pavesio et al. [27] First results after implantation in 67 patients showed good functional results and a cartilage repair in arthroscopic evaluation. Later on Gobbi et al [28] could support these results with own clinical data. Up to 5 years after transplantation they found statistically significant improvement in subjective and objective health scores. Interestingly a direct comparison of cartilage defect reconstruction by matrix-accociated chondrocyte transplantation with Hyalograft C and the conventional technique of simple microfracturing of the subchondral bone in the defect in two groups with similar injuries showed satisfactory clinical outcomes in both groups at 5 years after surgery. The benefit of chondrocyte transplantation with Hyalograft C was only marginal in clinical evaluation scores though statistically significant.[29]

The difficulty of porous solid scaffolds for tissue engineering is the homogenous distribution of seeded cells and the nutrition in central parts of larger implants.[30] A second disadvantage is the need of an operative treatment for implantation of the scaffolds. The clinical and biological restrictions led to the investigation of gelatinous materials for tissue engineering. The idea to mix natural derived polymer solutions with cell-suspension and cross-link it in the defect was tracked by some groups.[31] Bovine chondrocytes mixed with a fibrin glue showed 6 to 12 weeks after subcutaneous implantation in nude mice a well formed cartilaginous matrix and the presence of glycosaminoglycan production and an active proliferation by biochemical analysis of DNA content and histological investigation.[31] In adult horses the resurfacing of large artificial cartilage defects in the knee joint with mixture of allogenous chondrocytes and fibrin glue led to improved filling and significantly enlarged contents of specific proteins (glycosaminoglycan, chondroitin sulfate and dermatan sulfate) in comparison with empty defects in the contralateral knee joint. Histological staining revealed differentiated chondrocytes in the middle and deeper zones of the implant with a cellular pattern resembling hyaline cartilage.[32]

Collagene as main natural ECM component was used by Taguchi et al for encapsulation of chondrocytes. The authors used an alkali-treated collagen (AlCol) derived from pig skin and crosslinked it with a PEG-based 4-armed star polymer (4S-PEG). The crosslinker 4S-PEG (pentaerythritolpoly(ethyleneglycol)ethertetrasuccinimidylglutarate) with branched poly (ethyleneglycol) chains bears active ester groups and reacts under physiological conditions (pH 7.4, temperature 37 °C) thus preventing cell injury. The DNA content remainned constant within three weeks of in vitro culture indicated viable chondrocytes and a good biocompatibility of the modified collagene. The GAG content constantly increasing with culture time suggests that the cells produce physiologic cartilage proteins in the scaffold although for other important ECM components like collagene II and aggrecan only the appearance of mRNA could be proven but not a production of proteins.[33]

The modification of PLLA/PLGA blends with crosslinked type II collagene displayed better outcomes in vitro than the

synthetic polymers alone. In 3D cultures of porcine chondrocytes the collagen –modified scaffolds showed an enlarged cell number and higher contents of GAG and collagene. Cell proliferation and matrix production reached the value of native cartilage between four and six weeks of cultivation. In modified scaffolds chondrocytes maintained their cartilage-like phenotype and started the ECM production at the beginning of the culture period.[34] These effects had been allocated to excellent cytocompatibility of crosslinked type II collagene and its functional role in maintaining chondrocytes physiologic matrix production as reported earlier by the same group.[35] The implantation of these scaffolds seeded with 10^6 chondrocytes/ml into cartilage defects of femoral condyles proved the superiority of collagene II dotation of the polymer blends in New Zealand White Rabbits: While PLLA/PLGA or Collagen II alone did not result in defect healing and provoked persisting inflammatory reaction the combination of synthetic polymer blend and the crosslinked collagene II led to macroscopic complete defect repair. Histological examination after 6 months after surgery revealed cartilage-like repair tissue with round cells included in lacunae.[34]

The blend of porous PLGA with a diblockcopolymer, PLGA-PEG-NH_2 led to surface exposed reactive amine groups used for immobilization of hyaluronic acid by Yoo et al. [36] Hyaluronic acid, one of the major components of the extracellular matrix in connective tissues, is a natural polysaccharide containing N-acetyl-d-glucosamine and d-glucuronic acid. It is the main lubricant in synovial fluid and is known to interact with chondrocytes via surface receptors. The surface receptor CD44 is known to trigger a signaling pathway after binding to extracellular hyaluronic acid that retains the original phenotype of chondrocytes.[37] The modification of PLGA with hyaluronic acid resulted in a threefold increase of DNA content in comparison with unmodified PLGA after four weeks of incubation with chondrocyte culture. The content of GAGs and total collagene was significantly increased and a higher expression of collagen type II mRNA could be proven in RT-PCR. Histological staining supported these findings with higher collagene II content and a greater prevention of typical phenotype on scaffolds modified with hyaluronic acid.[36]

For long term is well known that cells are sensitive for surface topography in microscopic and range such as grooves, ridges and pores. Contemporary studies brought first insights of nanoscale influence on cell adhesion, orientation, motility, regulation of surface proteins, formation of the cytoskeleton, and intracellular signaling pathways. The cells are influenced not only by the range (typically 5 nm to micrometer range) and shape of topographical changes but also by their distance and symmetry.[38] The fibres of the ECM and basement membranes in human bone are characterized by typical nanometer dimensions with diameters between 10 and 300 nm as well as the HA mineral crystals with 4 nm length.[39] They represent the physiologic environment of cells with grooves, ridges interconnecting pores, mineral crystals in case of bone tissue (hydroxyapatite) and chemical binding sites for docking of specific anchor proteins at the outer cell membrane (integrins). The adherence of cells on a biomaterial surface and their further differentiation depend on their own origin on one hand and the nanotopography of the material on the other. The reaction of a cell type to its substrate is very specific and cannot be simply applied to other cell types. For the first contact with a materials surface or with each other cells may quickly form tiny filopodia and microspkies. Nanoscale topography may influence this formation process and therefore the attachment and the range of spreading, conformation of the cytoskeleton and further proliferation. The cytoskeleton is formed and orientated depending on grooves and ridges of the substrates surface and a change in the surface alone may cause different reactions of the same cell species. The figure below

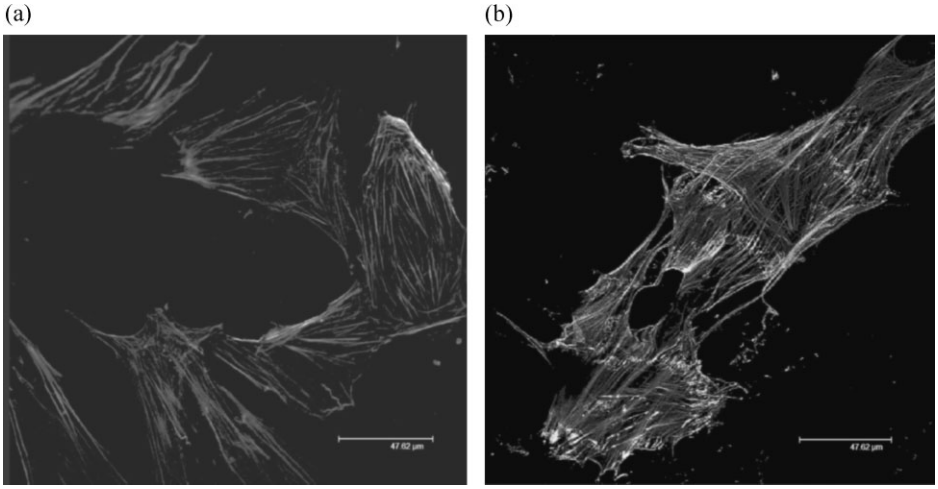

Figure 2.
Orientation of the cytoskeleton of osteoblasts depending on the surface topography of the substrate: a) a regular and more parallel pattern on smooth polymer culture membranes (polycarbonate) and b) a typical stretching of the cytofilaments between anchor-points elevating from the surface. Laser scanning microscopic pictures, immunohistological staining of actin filaments.

illustrates the different shape of primary osteoblasts on a microporous titanium surface and the smooth culture dish (Figure 2).

Concluding from the above mentioned findings nanostructured materials should provide the best conditions for the formulation of biomaterials by mimicking natural dimensions of the ECM environment. Pattison et al. could show that already the introduction of nanoscaled roughness into scaffold pore walls increased cell attachment, cell proliferation and the expression of matrix proteins.[40]

Nanofibres with diameters from one to several hundred nanometers can be produced with the simple and inexpensive technique of electrospinning from many synthetic or natural derived polymers. Electrospun nanofibre tissues have an extremely high and interconnective porosity allowing a free diffusion of gas and nutrients and allow controlled fibre geometry for guidance of cell orientation. The minute amount of material Aand dthe large interior surface (high surface to volume ratio) enhances degradation and resorption. They can imitate physiologic patterns orientation and diameter of ECM fibrils. Synthetic polymer materials offer a variety of technical modifications with from tailored mechanical properties to chemically defined position of side groups and binding sites. The resorption kinematics can be simulated in vitro and influenced by chain length, branching, crosslinking or chemical modification. Natural derived polymers (fibrin, chitosan, starch) represent the physiologic environment of living cells with very good biocompatibility, sophisticated steric structure, sometimes bipolar electric properties (collagene) and often specific pathways for their resorption and scavenge. But the process of manufacture and maintenance of their physical and chemical properties during extraction and remodelling is much more difficult and challenging than the manufacture of synthetic polymers.

Poly(ε-caprolactone) (PCL) was used for the production of three-dimensional electrospun nanofiber constructs by Lit et al. [41] PCL a semicrystalline biodegradable poly-hydroxy polyester shows slower degradation kinetics than PLGA or PLA commonly used as biomaterials. Mechanical

and degradation behaviour can be regulated by blending or copolymerization to modify its biological properties. PCL thus could serve as material for drug delivery systems or matrix material when slow drug release or prolonged presence of an artifical matrix will be necessary. The authors fabricated randomly distributed PCL - nanofibers with a mean diameter of 700 nm at the surface of slides and cut the mat into squares of 1 cm side length and 1 mm in diameter. The scaffolds were sterilized by ultraviolet irradiation and an amount of 4×10^5 fetal bovine chondrocytes was seeded onto the surface. Cell proliferation assays, immunofluorescence staining and common histological staining with Alcian Blue were performed at days 7, 14 and 21 to evaluated cell growth and differentiation. As result the expression of cartilage-specific genes indicated that primary bovine chondrocytes proliferated and maintained their differentiated phenotype on the PCL scaffolds. In contrast controls seeded on tissue culture polystyrene (TCP) remained in a dedifferentiated state with a lack of specific gene expression. In serum free culture medium the RT-PCR analysis proved the mRNA expression levels of collagen types II and IX, aggrecan, and cartilage oligomeric matrix protein (COMP) after 21 days. The mRNA expression of three integrin receptors mediating the cell–ECM interaction ($\alpha5$, αV, and $\beta1$) could be found to comparable levels each time of evaluation indicating a differentiated cell type. In proliferation assays of chondrocyte cultures a consistent increase in cell number was found on the nanofibrous scaffold but cell proliferation rate was reduced in comparison with cell growth on TCP. In confocal laser scanning microscopy significant differences in cell shape and cytoskeletal organization had been observed between nanofibrous scaffolds and those on TCP: While cells on TCP had a flattened, fibroblast-like morphology with long intracellular actin fibre bundles (stress fibers) extending throughout the entire cytoplasm the chondrocytes on nanofibrous scaffolds formed a round three-dimensional morphology with less cytoplasmic spreading. Short actin filaments were only randomly distributed throughout the cytoplasm of the spherical cells without larger stress fibres. The production of sulfated proteoglycans at the surface of the cell–PCL nanofibrous composite was more extended compared with control cultures on TCP in Alcian Blue staining.

The authors used the same PCL nanofiber scaffold to host adult mesenchymal stem cells (MSC) and found the material to support in vitro chondrogenesis. MSC represent pluripotent cells with the ability to transform into cartilage, bone, muscle, fat or bone marrow stroma. Today a variety of biological factors are known guiding the process of cellular differentiation. For induction of chondrogenesis a 3D culture with high cell density and intimate cell-cell contacts was found to be a prerequisite in studies with cell pellet culture.[42,43] The MSC underwent a chondrogenic differentiation in the PCL nanofiber scaffold during culture in serum free medium supplemented with TGF-$\beta1$. Rounded cells with morphological appearance of chondrocytes and the formation of ECM had been observed both in the cell pellets and in the nanofiber scaffolds after 21 days of breeding. The PCL nanofibres were in intimate contact with the cells and ECM. The detection of cartilage specific mRNA revealed the expression of Collagen II, collagen IX and aggrecan. The production of GAG was higher in the nanofibre scaffolds than in the pellet culture during the first two weeks.[44]

Nanofibre materials of poly(DL-lactide-co-glycolide) (PLGA) with different ratios of lactic and glycolic acid were tested by Shin et al. [45] for cultivation of procine chondrocytes. They found ECM formation and cell proliferation in nanofibre scaffolds superior to membrane shaped scaffolds. The application of hydrostatic pressure increased the amount of ECM and the number of cells. The nanofibre scaffolds had mechanical properties similar to skin tissue and tensile modulus was only slightly lower than those of human cartilage.

Scaffold Materials for Tissue Engineering of Bone

Bone represents a highly organised tissue with sophisticated architecture at macroscopic, microscopic and nanoscopic level. The vast majority of human bones belong to highest level of structural organisation: to the lamellar bone. This type is characterized by tiny sublayers of more or less parallel aligned collagene fibrils. The fibre direction is alternatively changing about 90 degree in each layer thus resulting in a complex rotating plywood-like architecture. The collagen fibrils become mineralized at chemically determined sites by formation of hydroxyapatite nanocrystals which stiffen the organic matrix. With respect to materials science bone can be regarded as a mineral reinforced fibre texture consisting of a main organic fibre matrix providing elasticity and tiny mineral crystals providing stiffness and load resistance. The lamellar fibre arrays form cylindrically shaped osteons in secondary bone that are aligned along the longitudinal axis of the bone in the direction of the main load transmission. The osteonal structure increases load resistance and determines the fracture behaviour of the material. A constant remodelling of the tissue renews the material, ensures an adjustment to the actual biomechanical situation and repairs crack damages. This bone architecture explains its superior mechanical properties allowing large load bearing with a relatively small amount of material.[46] The substitution of bone with synthetic materials is therefore a challenge to material engineers and unless unquestionable advantages in the past decades there is no fully satisfying alternative to natural bone transplant available today. However, ceramic materials based on calcium and phosphates became widely used in clinical medicine for the treatment of bone defects. Especially hydroxyapatite (Ca_{10} $(PO_4)_6(OH)_2$, HA) proved its excellent biocompatibility and osteoconductive properties, i.e. it serves as a guide rail for formation of new bone. But low tensile and bending strength, the brittleness of macrostructured HA implants and the lacking resorbability as a prerequisite for bone remodelling limit medical applications. HA cannot be used for the repair of load-bearing parts of the skeleton and its in vivo function is limited to serve as a filler or as surface coating of metal implants.[47] Many approaches tried to overcome this disadvantages of massive HA implants. The idea to minimize the dimension of HA led to nanocrystal shaped biomaterials applicable in aqueous suspension by injection. But both experimental results in vivo and clinical outcome of first application revealed serious side effects and poor resorbability of nanocrystals.[48,49] A promising alternative is seen in composite materials. The incorporation of HA or comparable mineral compounds into polymer nanofibers create biomimetic scaffolds for substitution of bone and improves the mechanical properties. Various combinations of HA and biocompatible polymers had been developed and investigated in vitro until today.[50–52]

A composite of polycarbonate (PC) and HA with a crystal size of 10–25 nm in diameter and 60–80 nm in length was manufactured by Jianguo et al [53] by injection-molding. For in vitro studies specimens of $10 \times 10 \times 1$ mm had been incubated with marrow-derived mesenchymal stem cells of neonatal rats for 11 days. A cell proliferation assay could proof a significantly higher cell number after one day compared with controls (native culture wells) but no differences from day 4 to day 11 of incubation. The authors concluded that the composite material has no negative effect on the cell morphology, viability, and proliferation. For in vivo evaluation HA/PC composites were implanted into thigh bones of eight New Zealand White Rabbits. The animals were sacrificed 4 and 16 weeks following implantation. In histological studies new formed bone was observed between implants and the surrounding host bone remained without signs of inflammation or implant rejection. The new bone is described with a density similar to normal but it remained immature up to 16 weeks after surgery.

Electrospinning of gelatin with HA precipitates created fibres with homogenous distribution of nanocrystals without seggregation. Subsequent crosslinking resulted in stable membranes with irregular fibre orientation. The incorporation of HA crystals improved the mechanical properties of the gelatine reaching a maximum at 20% of mineral content. In mechanical testing the stress-strain pattern of the composite was quite similar to that of pure gelatin but tensile strength and elastic modulus were improved with a reduced strain at failure. The bone derived cell line MG63 produced significantly more alkaline phosphatase (an important marker of bone forming cells) on HA composites than on pure gelatin fibres. A composition gradient was introduced in gelatin/HA nanofiber membranes to adjust hard and soft tissues for more effective guided regeneration.[54]

The differentiation of MSCs on nanofibrous scaffolds of degradable PCL had been assessed by Shin at al.[55] MSCs were harvested from bone marrow of neonatal rats and precultured for expansion. Cells were seeded PCL mats and kept in culture with osteogenic medium in a rotating bioreactor for 4 weeks. The cell-polymer constructs were subsequently grafted on the omentum majus of adult female Lewis rats. One week after surgery stem cells had migrated into the interior of scaffold and produced extracellular matrix. In the inner and outer parts of the scaffold collagene I could be found by light microscopy. After 4 weeks the implants became harder and their size was unchanged. Light microscopy revealed a mineralized bone-like tissue with scattered osteocyte-like cells.

Electrospun nanofiber scaffolds from silk fibroin containing bone morphogenetic protein 2 (BMP-2) and/or nanoparticles of hydroxyapatite were manufactured by Li et al [56] to cultivate human bone marrow-derived mesenchymal stem cells (hMSC). The sensitive protein BMP-2 survived the in aqueous-based electrospinnig process in bioactive form. After 3 weeks of culture in a osteogenic culture medium BMP2 containing scaffolds enhanced higher calcium deposition and the transcript levels of bone-specific markers (bone sialoprotein-II, collagen type I) in comparison with the controls. The scattered HA particles in the nanofibres improved bone formation. The results indicating that electrospun silkfibroin-based scaffolds seem to be potential candidates for bone tissue engineering.

The combination of starch/poycaprolactone (SPCL) (30/70 wt%) was used by Tuslakoglu et al [57] for fabricatin of scaffolds with 70% porosity. Randomly distributed, electrospun nanofibres with an average diameter of 400 nm of the same material were inserted and form nanobridges between the microfibres in SEM investigation. The culture of human osteoblast-like cells (SaOS-2, cell line, European Collection of Cell Cultures) and rat mesenchymal stem cells showed differences of the nano/micro fiber combined scaffolds in comparison with the control fiber mesh scaffolds. Morphological assessment revealed different cell shape and morphology and both cell types showed increasing metabolic activity and growth rates when seeded on combined scaffolds. The activity of the osteoblast enzyme alkaline phosphatase was found to be higher on combined scaffolds after 7 and 14 days of culture.[57]

The combination of parallel aligned starch–polycaprolactone microfibrenets produced by rapid prototyping (RP) and electrospun polycaprolactone nano-motifs resulted in 3D scaffolds with interesting properties. RP microfiber scaffolds were reported to need high cell numbers to reach satisfactory cell density for tissue formation. The minimal pore size reachable with rapid prototyping is relatively large as compared to the dimension of a cell. Seeded cells penetrate through the pores of RD scaffolds and adhere at the culture dish.[58] Randomly distributed nanofibres had been interlaced by to function as anchor structures with adequate fibre dimension for cell adhesion.[59] The integration of nano-motifs diminished the scaffold porosity to 68% but a fully interconnected pore system could be main-

tained allowing diffusion of gas and nutrients. Human osteoblastlike cells (SaOS-2 cell line) had been seeded onto the scaffolds. During the primary phase of cell attachment a spinner flask bioreactor was used as a dynamic system to gain better penetration of the cells. The evaluation with SEM micrographs shows the homogenous cell distribution throughout the scaffold and a preferential adhesion of the cell at the nanofibre components. The interlaced nanofibre obviously acted as cell entrapment structure in the system. While there was no difference in the cell viability measurement between RD microfibre alone and the scaffolds containing additional nanofibres a significant increase of cell proliferation and protein synthesis as a sign of maturation could be proven in the latter.[59] These very important results clearly demonstrate that microfibers may have a potential function supporting the mechanical stability of polymer scaffolds but the mainly important structure for cell entrapment must have a nanometer dimension.

Topographical features influence the physiological behaviour of osteoblastic cells like adhesion and spreading. They can stimulate osteocalcin and prostaglandin synthesis[60,61] and increase the activity of alkaline phosphatase.[62] But also topographical factors like fibre geometry and fibre diameter can influence cell density. Badami et al [63] could show in PDLLA, PLLA, PEG-PDLLA and PEGPLLA electrospun nanofibres that osteoprogenitor cells not only adhere and proliferate but in the presence of osteogenic factors cell density on fibers was equal to or greater than that on smooth surfaces. Osteoprogenitor cells attached to electrospuin fibres of 2.1 μm diameter were observed to extend lamellapodia along individual fibers and exhibited a higher cell aspect ratio than cells cultivated on smooth surfaces. The study substantiates that biomaterial topography can provoke contact guidance and is able to regulate spreading, orientation and proliferation of osteoprogenitor cells.[63] In PLGA molds containing carbon fiber formulations of either nanophase (i.e., dimensions less than 100 nm) or conventional (i.e., greater than 100 nm) dimensions significantly increased rates of osteoblast adhesion were observed as early as one hour after seeding.[64]

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