

Nanostructuring of Biomaterials – A Pathway to Bone Grafting Substitute

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Abstract

Background: The bone substitute NanoBone[®] consists of nanocrystalline hydroxyapatite embedded in a highly porous matrix of silica gel. It promotes the healing of bone defects and is degraded by osteoclasts during bone remodeling. The present study investigates the interactions of NanoBone[®] with bone tissue.

Methods: Granules of NanoBone[®] were implanted in defects of critical size in the mandible of minipigs. Samples were taken after 5 and 10 weeks and demineralized. The composition of the implanted granules was analyzed by means of transmission and scanning electron microscopy and EDX. Enzyme- and immunohistochemistry was used to investigate organic components of NanoBone[®] granules that arised after implantation in the host.

Results: EDX demonstrated that 5 weeks after implantation the silica gel was degraded and replaced by an organic matrix. Ultrastructurally, the matrix appeared amorphous with only single collagen fibrillae. PAS-staining indicated the presence of carbohydrates. Immunohistochemically, the bone proteins osteopontin, osteocalcin and BMP-2 were found as constituents of the new matrix. Alkalic phosphatase activity was located in osteoblasts and newly formed bone on NanoBone[®] and focally in particles. Osteoclasts with ruffled borders, sealing zones, and acid phosphatase activity were situated in resorption lacunae at granule surfaces not covered by new bone.

Conclusions: In vivo, the silica gel of NanoBone[®] is replaced by bone matrix glycoproteins with known functions in attraction, adhesion, and differentiation of bone cells as osteoblasts and osteoclasts. We assume that the deposition of these molecules supports the early phase of NanoBone[®] degradation by osteoclasts and promotes the production of new bone tissue.

Key Words

Bone graft substitute · Remodeling · Immunohistochemistry · Growth factors · NanoBone[®]

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Introduction

Nanotechnology provides new materials in the nanometer range with many potential applications in research and medicine. Due to their unique size-dependent properties nanomaterials, such as nanoparticles, offer the possibility to develop both new diagnostic and therapeutic tools.

A special feature of nanostructured materials in comparison to bulk material is the existence of an extreme high number of molecules on the surface. This is also the case in nanoporous materials. There, a very high inner surface can be found. If autologous molecules,

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e.g. from patient's blood during surgical operation, can be bound to the inner surface a new level of bioactivity is achieved. Biological cells are naturally larger than nanostructures of these biomaterials. Due to this structure the cells do interact only with the autologous molecules and not directly with the biomaterial. This characteristic offers new opportunities for bone grafting materials.

The biomaterial market is expanding rapidly in parallel with the higher demands for bone graft substitute materials. Since the golden standard, the autograft, is still preferred by many surgeons, the biomaterials for their development have to be highly bioactive, to possess osteogenic potential, to degrade easily in biological fluids and not to cause immunological inflammations. Therefore, a great variety of the specially synthesized calcium phosphate materials to be used as bone graft substitute materials exist. Although all these biomaterials are of the same family, there are differences between the product properties, e.g. crystallinity, porosity, phase purity, mechanical and thermal stability. The desired characteristic is dependent on the required application of the material.

The aim of this study was to investigate the interaction of the new synthetic bone grafting substitute NanoBone[®] with the autologous tissue. When the material was used as a bone grafting substitute, rapid healing of critical size defects was observed both in animal experiments and in human applications [1–7]. Once the defect has healed, the material was degraded almost completely. This degradation process was carried out by osteoclasts during remodeling of the new developed bone tissue [1–3].

Investigation of the interaction in the early phase of defect healing, and how the nanostructured silica gel matrix responds to autologous tissue is of special interest. Beside electron microscopy investigations of the biomaterial after 5 and 10 weeks in vivo, histochemical and immunohistochemical investigations were done.

Up to now only a few studies on bone graft substitutes are known where immunohistochemical research was done [7–9].

In this study alkaline phosphatase, osteopontin, osteocalcin and BMP-2 were analyzed by immunohistochemistry.

Alkaline phosphatase is a marker for mature osteoblasts and is synthesized by them. Probably it has a function in calcification of the matrix [10].

Osteopontin is a relevant bone sialoprotein. It has many functions in the early phase of osteogenesis and

differentiation of osteoblasts. Osteopontin controls not only the mineralization and adhesion of osteoblasts but also the differentiation and adhesion of osteoclasts. It is strongly bound to hydroxyapatite [11–14].

Osteocalcin is an osteoblast specific marker. It is more important for later processes of osteogenesis. It is deposited in the osteoid shortly before mineralization [13]. In vivo, osteocalcin and osteopontin are typical markers for the mineralization sequence [15].

BMP-2 belongs to the family of bone morphogenetic proteins within the TGF- β -family. Many BMP's are considered as potent osteogenetic factors. They stimulate osteogenesis in different types of cells. They were already applied in experimental and clinical therapies for bone regeneration [16–18].

Material and Methods

Bone grafting substitute: NanoBone[®] is characterized by nanocrystalline hydroxyapatite embedded in a matrix of silica gel (approx. 24% by weight) which is porous down to the nanometer size range.

Silica gel is an incomplete linkage of polysilicic acid. It is characterized by numerous open bonds, which are always SiOH groups or SiO-groups (depending on the

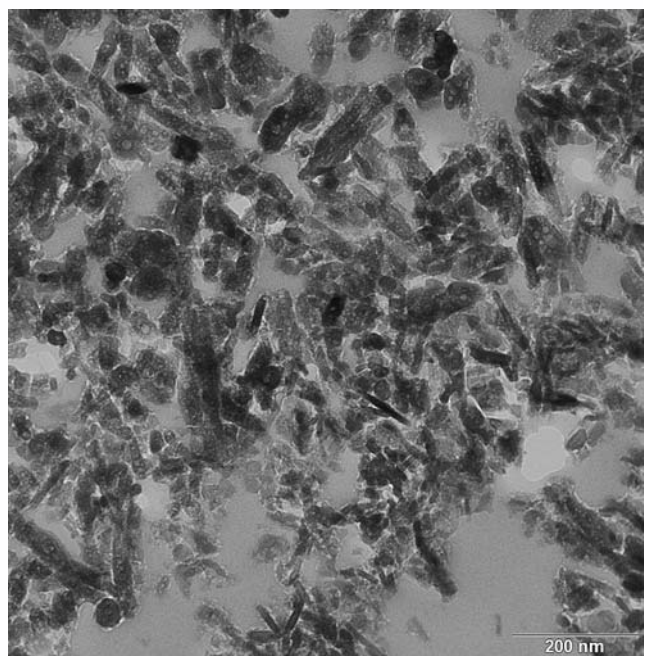


Figure 1. Transmission electron micrograph of the nanostructure of NanoBone[®]. The nanocrystalline HA is embedded in a matrix of silica gel (SiO₂) having interconnecting pores. The sample is a thin slice prepared by ultramicrotomy. Therefore it is embedded in epoxy resin and it is not possible to distinguish between epoxy and silica gel.

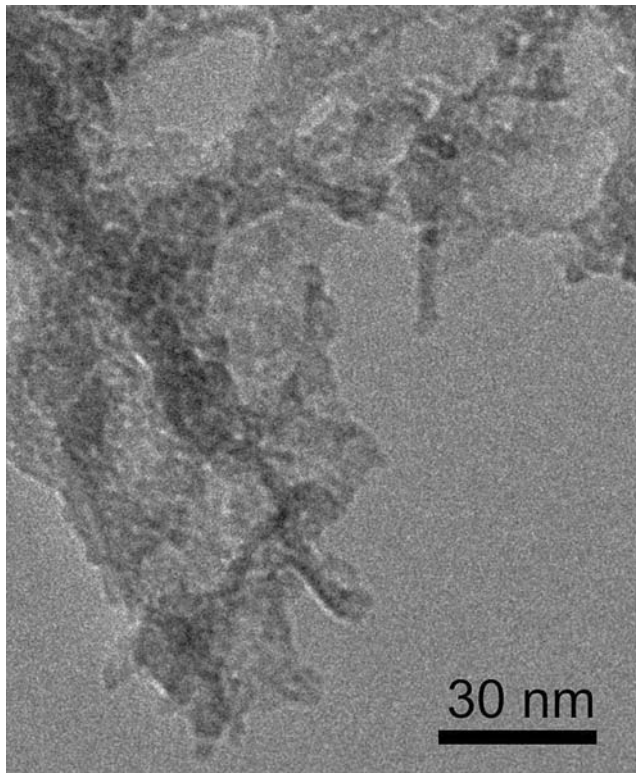


Figure 2. Transmission electron micrograph of the matrix of the silica gel after HA has been dissolved with EDTA. The maximum pore size distribution of the interconnecting pores is 20 nm. Picture taken at a break line.

pH value). Owing to these open bonds, the internal surface of the material is extremely large. This internal surface is $84 \text{ m}^2/\text{g}$ in size. The pores in the silica gel have maximum pore sizes ranging from 10 nm to 20 nm and are interconnecting. As a result, blood plasma and, thus, autologous proteins may penetrate into this open structure once the silica gel comes into contact with the patient's blood. The porosity of the material is 61%.

Figure 1 is a transmission electron micrograph of NanoBone[®] demonstrating its loose structure. In contrast, conventional calcium phosphate biomaterials are sintered. In that way the crystallites are strongly connected and form a dense texture.

Figure 2 shows a transmission electron micrograph of the matrix of the silica gel. In order to better represent the silica gel matrix, the hydroxyapatite was dissolved out of this matrix with the help of EDTA. As can be seen from the figure, the matrix is inhomogeneous to a size range of a few nanometers. The structure of the pores is well visible. To ensure a better contrast, the gel matrix was not embedded in epoxide but was represented at a break line. Since silicic acid has its

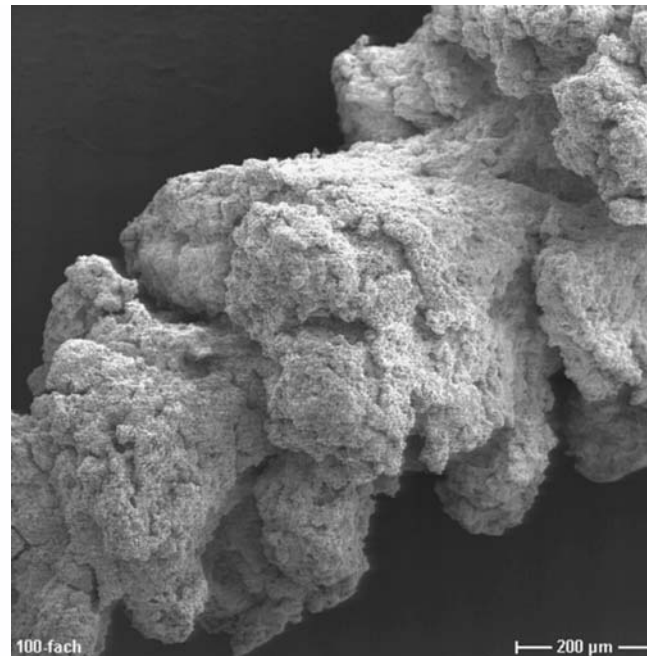


Figure 3. Scanning electron micrograph of a granule having a high surface roughness.

isoelectric point at a pH of 2 – that means that the surface is neutral at this pH value – the surface of the silica gel is charged negatively at a pH of 7, which is generally the pH of tissue. In other words, the surface is determined by SiO^- groups. If autologous proteins are entering into the pores together with the blood plasma, proteins with a positive charge are particularly retained. Despite the fragile structure of the silica gel, the material has a relatively high-pressure resistance of 40 MPa. To be able to use the material on patients, its pressure resistance must be sufficiently high; a low-pressure resistance poses the risk that the material might “crumble” – and be finally degraded by macrophages, resulting in complications if the fragments of the material are too large.

Figure 3 shows a scanning electron micrograph of a granule. A granule has the shape of a fir cone with a mean length of 2 mm and a mean diameter of 0.6 mm. The high surface roughness of the granule which ranges down to the micrometer size range is well visible.

Animal experiments: In the anterior mandibles of ten 1-year-old Goettingen minipigs (Body mass: 20–25 kg), perforating defects with the critical size of $> 5 \text{ cm}^3$ were artificially produced that would not heal spontaneously and were neither in contact with the oral cavity nor the teeth.

After intramuscular premedication with 1 mg atropinum sulfuricum (Eifelfango, Bad Neuenahr,

Germany) and 5 g/kg azaperon (Stresnil, Janssen-Cilag, Neuss, Germany), the animals were anesthetized intramuscularly with ketamine hydrochloride (Ketamin, Belapharm, Vercha, Germany) and 0.2 mg/kg midazolam hydrochloride (Dormicum, Hoffmann-La Roche, Grenzach-Wylen, Germany). Postoperatively, the minipigs were fed with a special dry food (Sniff-Spezialdiätfutter, Soest, Germany) and water at libitum.

Histology: The animals were sacrificed 5 and 10 weeks after implantation of NanoBone® and tissue samples from the former defect region were obtained. Samples were fixed in 4% phosphate-buffered formaldehyde for a minimum of 7 days before cutting transversally into blocks of about 4 mm thickness. The blocks were decalcified with EDTA and embedded in paraffin. Tissue sections of about 3.5 µm thickness were stained with hemalaunum and eosin (HE).

Histochemistry: Selected sections were stained with periodic acid Schiff (PAS) staining to localize glycoproteins in general. In order to identify osteoclasts, selected tissue sections were stained to demonstrate tartrate-resistant acid phosphatase (TRAP). In short, after TRAP-activation in tris-hydrochloride acid-buffer, the incubation was carried out in a solution containing a mixture of pararosaniline-hydrochloric acid with sodium nitrite, veronal acetate-buffer and tartaric acid and of naphthol-AS-biphosphonate with dimethyl formamide for 1 h at room temperature (rt). Thereafter, slides were rinsed, counterstained with Mayer's hemalaunum and mounted. All chemicals were purchased from Sigma (Deisenhofen, Germany).

Immunohistochemistry: After deparaffinization and rehydration, selected sections were processed for immunohistochemistry. In short, after incubation with primary antibodies against alkaline phosphatase (AP, polyclonal rabbit, Quartett, Berlin, Germany) diluted ready to use over night at 4°C, Bone Morphogenetic Protein-2 (BMP-2, polyclonal goat, Santa Cruz, USA) diluted 1:25 over night at 4°C, osteocalcin (monoclonal mouse, Takara, Shiga, Japan) diluted 1:1000 for 1h at rt and osteopontin (polyclonal rabbit, abcam, Cambridge, UK) diluted 1:200 for 1h at rt sections were washed in TBS. Secondary peroxidase labeled "envison" antibodies (DakoCytomation, Glostrup, Denmark) according to primary antibody species each (anti-rabbit, anti-goat, or anti-mouse) were incubated diluted 1:50 for 30min at rt. After rinsing, peroxidase activity was visualized using diaminobenzidine (DAB, Sigma, Deisenhofen,

Germany) yielding a brown staining product. Slides were counterstained with Mayer's hemalaunum.

Results

Figure 4 shows a histological section of a bone defect 5 weeks after the biomaterial had been implanted. It was made from a demineralized tissue sample and demonstrates a granule positioned in a newly formed bone tissue. Lacunae with osteoclasts can be seen in this granule. Using a scanning electron microscope, the composition of the remaining granule is to be determined with EDX.

Figure 5 shows the scanning electron micrograph of a granule from a demineralized tissue sample (a) along with the associated EDX spectrum (b). The demineralization process with EDTA does not attack the matrix of the silica gel.

According to the EDX spectrum, the granule that remains after demineralization, as shown in the histological section or in the scanning electron micrograph, is nothing but an organic material. Only a small percentage of sulfur can be detected in addition to the carbon peak.

This organic matrix was developed during the first 5 weeks after implantation. Transmission electron microscopic examinations were used to provide more detailed information about the structure of this organic matrix.

Figure 6 shows enlarged pictures of the granules. The typical organic matrix, which is more or less

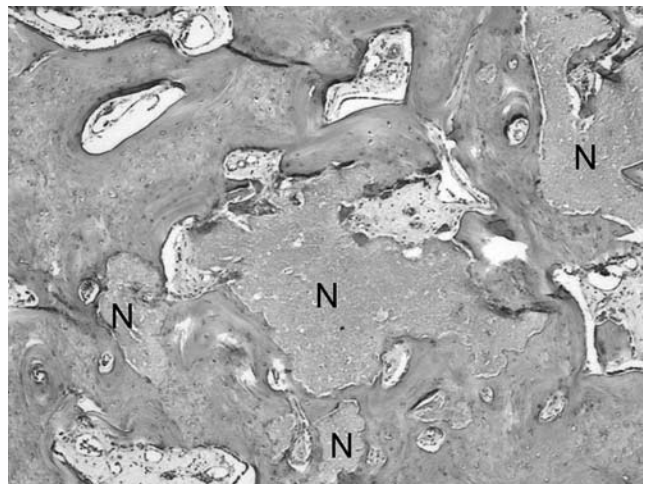
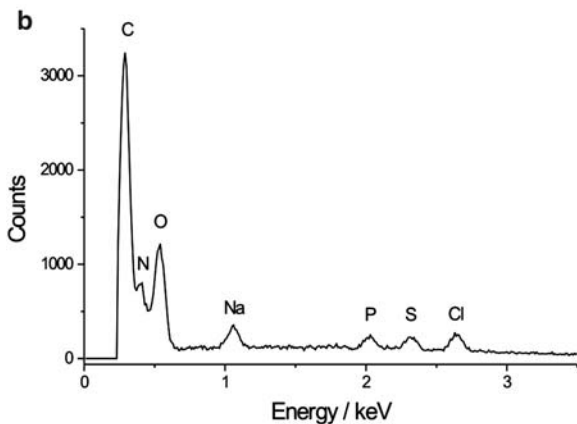
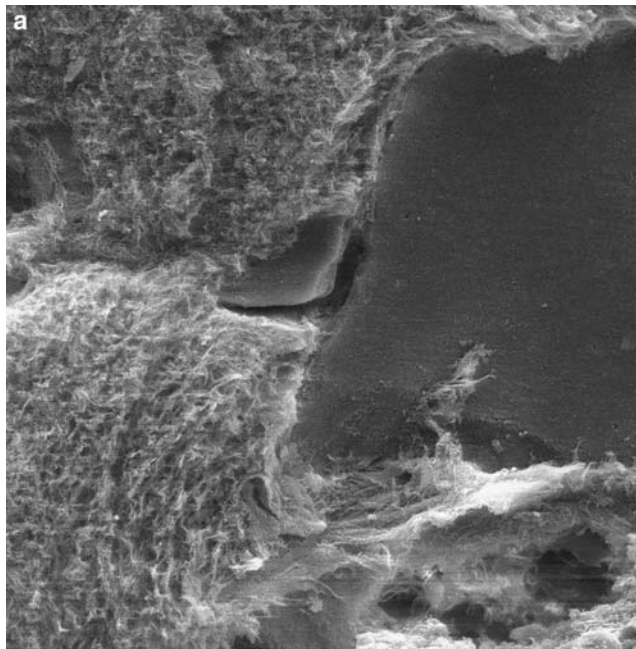


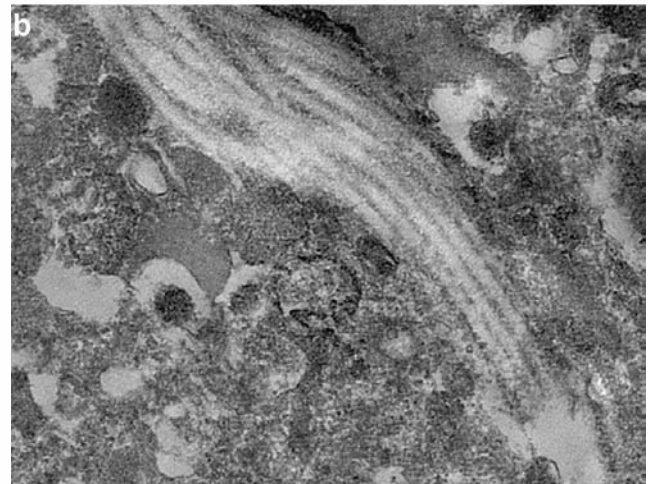
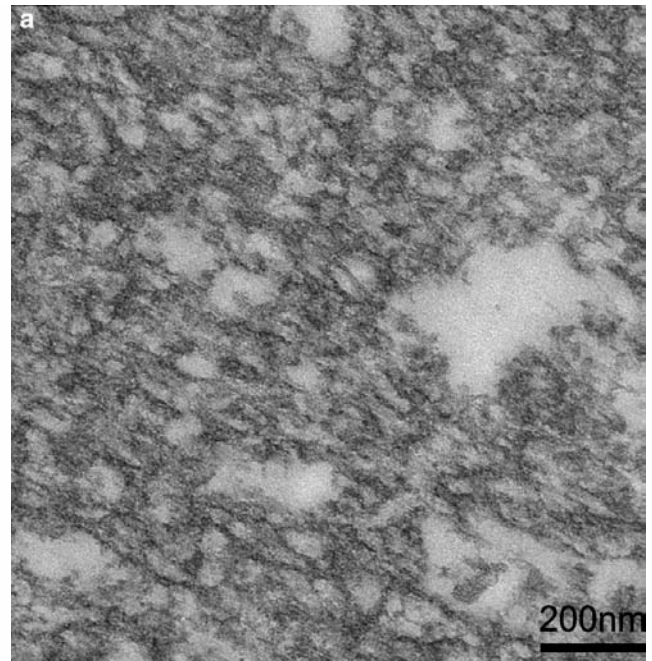
Figure 4. Micrograph of a demineralized histological section, stained with HE, from a sample 5 weeks after implantation of NanoBone. The NanoBone granule (N) located in the center of the micrograph is surrounded by newly formed woven bone. On its upper face osteoclasts are situated in resorption lacunae. Note that osteoclasts also resorb bone trabeculae contacting the granule.



Figures 5a and 5b. Scanning electron micrograph (a) of a demineralized granule in the tissue (5 weeks after implantation) and EDX spectrum (b) of the granule. Owing to the demineralization process, calcium phosphate cannot be detected any longer. It is not possible to detect silicon from the matrix of the silica gel, although the demineralization process does not attack SiO_2 . The granule contains nothing but organic constituents.

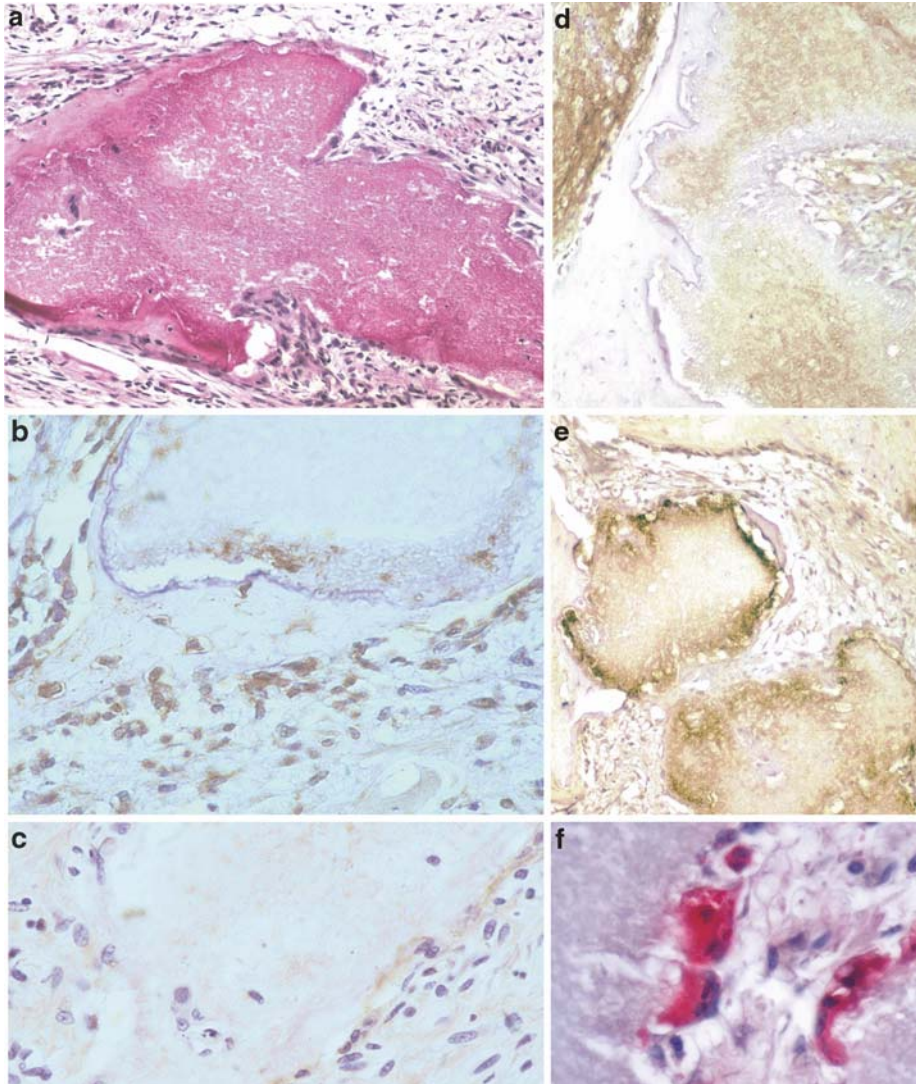
amorphous can be seen in Figure 6a. The samples are embedded in epoxy and have been contrasted (osmic acid and uranyl acetate), so the organic components are pictured dark. The light regions are holes in the organic matrix, caused by the hydroxyapatite crystals that were dissolved out by EDTA. There are isolated collagen fibrillae in the unstructured organic matrix, as shown in Figure 6b.

The following section presents the histochemical results.



Figures 6a and 6b. Transmission electron micrograph of the granule 5 weeks after implantation and after demineralization. The samples have been contrasted (osmic acid and uranyl acetate) and embedded in epoxy resin. There is the typical newly formed amorphous organic matrix, which contains holes representing the sites where HA crystallites have been dissolved out (a). The organic matrix contains isolated collagen fibrillae (b).

Figure 7a shows a typical example of PAS staining. There are similar findings after 5 and 10 weeks. A weak to moderate PAS staining of connective tissue and cells including osteoclasts and osteoblasts can be seen. A focal PAS staining of bone matrix (preexistent and newly formed bone, e.g. cement lines) as usual is shown. NanoBone[®] responds PAS-positive especially at the periphery and also at the border between NanoBone[®]



Figures 7a to 7f. a) Micrograph of a demineralized histological section from a sample 5 weeks after implantation of NanoBone® with PAS staining for the detection of glycoproteins and glycolglycans in the organic matrix in the granule. Osteoblasts secrete osteoid directly on the granule. b) AP staining (brown) shows active osteoblasts secreting osteoid. Positiv areas are at the surface of the granule. c) BMP-2 immunostaining (brown). NanoBone® weak staining the surface of the granule. d) Immunoreactivity toward osteopontin in the demineralized section (5 weeks after implantation). The brown coloration shows that osteopontin has a higher concentration in the center of the granules. e) Immunoreactivity towards osteocalcin in the demineralized section (5 weeks after implantation). The brown coloration indicates osteocalcin which can be detected at the surface of the granule. f) Micrograph of a demineralized histological section from a sample 5 weeks after implantation of NanoBone®, stained for acid phosphatase activity. Osteoclasts cover the surface of a granulum. Near the upper border of the micrograph two osteoblasts are in contact with the biomaterial.

and newly formed bone tissue. Newly formed bone tissue (osteoid) also reacts PAS-positive. Strong PAS-positive NanoBone® residues can be seen within the newly formed bone. Especially in sections from 10 weeks, there is a PAS-positive “lamina limitans” on the surface of newly formed bone beneath osteoblasts (newly formed undecalcified osteoid).

The PAS staining helps to detect carbohydrate-containing components, such as glycoproteins, mucins and glycogen, by means of periodic acid and Schiff reaction. The entire organic matrix, which has remained after demineralization, shows positive reactions. This figure shows very clearly that the osteoblasts are secreting osteoid on the surface. The bottom side shows osteoclasts, which are arranged in lacunae in the biomaterial. The sulfur peak in the EDX spectrum shown

in Figure 5b may be assigned to these carbohydrate components. Since the charge of these molecules is negative, it cannot be assumed that they were directly added to the silica gel surface, which is also charged negatively.

Immunohistochemical results will follow below. The AP staining is shown in Figure 7b.

After 5 and 10 weeks: Original bone: positive immunostaining for osteoblasts and osteocytes. NanoBone®: focal granular net-like immunostaining in the center and periphery of particles. The osteoblasts located on the NanoBone® particles are also positive. There are also osteoblasts and osteocytes in newly formed bone on NanoBone®. Osteoclasts are not stained.

An example for the results for BMP-2 is shown in Figure 7c.

After 5 and 10 weeks: Weak immunostaining in original bone focally in matrix and in connective tissue of bone. NanoBone®: weak immunostaining in some of the particles. The moderate immunostaining of osteoblasts is located directly on NanoBone®. Later also osteoblasts are positive located on newly formed bone spiculae around NanoBone® particles. There is no immunostaining of the osteoclasts.

A typical result with the osteopontin staining is shown in Figure 7d.

After 5 weeks: moderate to strong immunostaining of connective tissue, also between NanoBone® particles, staining of vessel walls; weak to moderate staining of NanoBone®. The matrix is more in central parts of

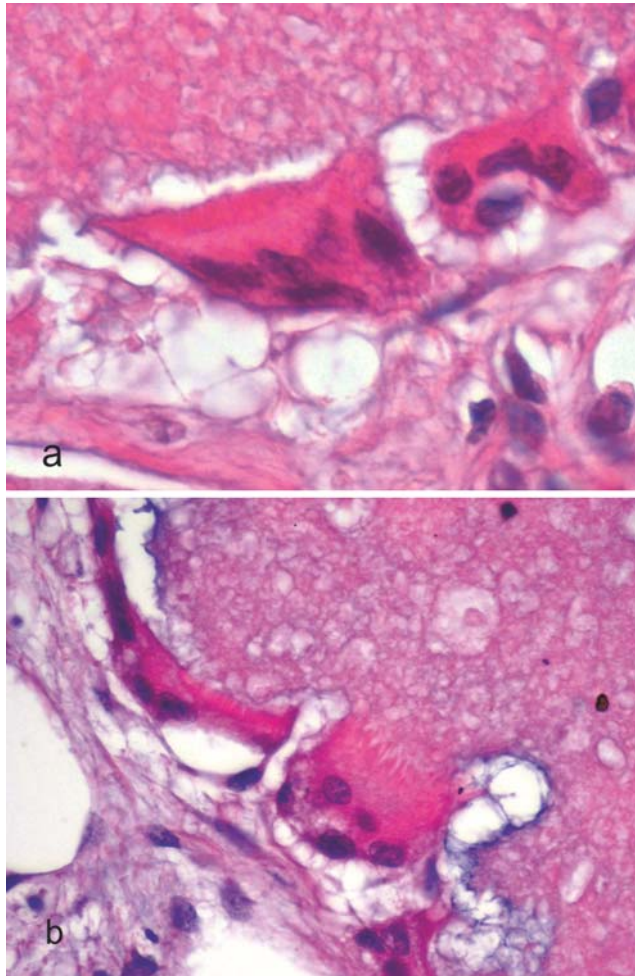


Figure 8. Microphotograph of a demineralized histological section stained with HE from a sample 5 weeks after implantation of NanoBone®. Osteoclasts with visible ruffled borders (b) and sealing zones (a) are located in shallow resorption lacunae on the surface of NanoBone®.

particles. The newly formed bone tissue matrix is focally immunoreactive.

After 10 weeks: Same staining pattern as in 5-week specimens, but weaker.

Figure 7e documents the immunoreactivity towards osteocalcin.

After 5 weeks: The original bone and the newly formed bone: immunostaining of bone matrix, especially in spongy bone trabeculae, cement lines, periosteum, osteoblasts and bone connective tissue. NanoBone® particles: moderate to strong immunostaining mainly at periphery of particles, only moderate to weak staining in the centers, weak or no staining in the osteoblasts on NanoBone®.

After 10 weeks: There is the same staining pattern as that for 5 weeks, but weaker.

Here, it is striking that the granules show a stronger reaction on their surface than in their inner region. That means that osteocalcin is concentrated in the outer layer of the granule by approximately 100 µm.

It is striking in all histological sections that a great number of osteoclasts is active on the surface of granules.

Figure 8 shows a typical osteoclast with ruffled border on a granule where the sealing zone toward the material can be seen. Histochemically, the activity of the osteoclasts on the surface of the biomaterial can be detected by means of acid phosphatase, thus furnishing unequivocal proof of their existence. Figure 7f shows an example.

Discussion

NanoBone® is characterized by nanocrystalline HA that is embedded in a highly porous matrix of silica gel. As has been detected by means of scanning electron microscopy and EDX, this silica gel matrix is degraded in vivo within a short time period without the granule losing its outer shape. This rapid degradation process is unusual because the solubility of SiO₂ in H₂O is very low at a pH of 7, so that this degradation cannot be a mere solubility process. Using demineralized histological sections, it was demonstrated that HA is tied in the granule by an organic matrix which has formed in vivo in parallel with SiO₂ degradation. The transmission electron micrograph shows that the organic matrix is unstructured and that there are only a few isolated collagen fibrillae.

Different staining methods (histochemical and immunohistochemical methods) were used to iden-

tify various organic components of the newly formed matrix.

The positive PAS reaction speaks for the presence of glycoproteins and proteoglycans that are also present in the extracellular matrix of bone. This reaction must be considered as an indication of a general inclusion of matrix components. Particularly the strong PAS reactivity at the periphery of the NanoBone[®] particles or at the interfaces between NanoBone[®] and the new bone that has been deposited on the latter speak for the fact that organic components are especially of functional importance here.

Specific immunohistochemical examinations then showed immunoreactivity for osteopontin and osteocalcin, matrix components approximately corresponding to the PAS-positive areas. The detection of these two components in NanoBone[®] means that they are included here. Osteopontin can be bound to the nanocrystalline hydroxyapatite. A primary source of these components may be blood serum that penetrates the bone replacement material after application and transports numerous bone metabolism components [19].

A time pattern can also be imagined here. Since it is more strongly located in the center of the particles, osteopontin was probably included at an earlier point than osteocalcin, which was preferably detected at the periphery. It might, however, also be possible that both components are secreted by fibroblasts and osteoblasts in the further course. Both components are promoters of osteogenesis.

Osteopontin also acts as an adhesion protein for osteoclasts and controls their resorptive activities [20, 21]. That means that osteopontin acts as an "attractor" for osteoclasts or their precursor cells in NanoBone[®], thus allowing a first step toward remodeling processes in NanoBone[®]. For example, osteopontin is absolutely necessary for the resorption of ectopic bone in the rat model (bone discs implanted in muscle) [21].

The preferred localization of osteocalcin at the periphery or at the interface between NanoBone[®] and newly formed bone tissue indicates that, in an early phase, it might play a role in the attraction, adhesion and further differentiation of osteoblasts as well as in the early phases of matrix secretion.

Immunoreactivity for alkaline phosphatase shows that osteoblasts are active both on NanoBone[®] and on the bone trabeculae having formed on NanoBone[®].

Immunoreactivity for BMP-2 is also interesting. They occur primarily in and around osteoblasts and

indicate a potential secretion through these cells. An endogeneous expression and secretion of this potent osteogeneous factor may be induced by the biomaterial, all the more so since BMP-2 also highly regulates all essential bone proteins [16].

It has long been known that osteoblasts generally react with changes in their gene expression and secretion on different surfaces [22].

The decreasing immunoreactivity for osteopontin and osteocalcin ten weeks after NanoBone[®] implantation indicates that these components play a more important role in the early phases of NanoBone[®] remodeling.

Within 5 weeks, the completely synthetic inorganic biomaterial changed into a material which is similar to the extracellular bone matrix. Contrary to bone, however, the organic matrix of NanoBone[®] granules contains almost no collagen fibrillae.

For that reason, this material cannot have the same tensile strength as natural bone. In its mechanical properties, it is not comparable with natural bone.

The further degradation of the bone replacement material is accomplished by osteoclasts. This means that the degradation is associated with the new formation of natural bone, because the activity of the osteoclasts is controlled through osteoblasts which form new tissue. Bearing in mind that the silica gel matrix in the NanoBone[®] granule has been replaced by an organic matrix, it is now also understandable how the degradation process of the biomaterial through osteoclasts can be accomplished at all.

If the granule consisted of original material, i.e. if there were hydroxyapatite crystallites in a highly porous silica gel matrix (61% porosity), a reduced pH value on the material side of the osteoclasts would not have any effect because the high porosity would cause rapid diffusion of the protons. In other words, it would not be possible to reduce the pH on the bottom side of the osteoclasts to such a low value so that hydroxyapatite is dissolved. It is nothing but the organic matrix which allows the material to be degraded through osteoclasts and, thus, to participate in the remodeling process of natural bone. An explanation of the high osteoclastic activity may be the fact that, although it is similar to the extracellular bone matrix, the newly formed organic matrix does not have the same high mechanical strength (owing to the missing collagen fibrillae) and is, thus, degraded in the same manner as low-grade bone.

Since the conversion of the silica gel matrix into an organic matrix is completed after 5 weeks, the animal experiments presented here do not allow any statement on the conversion process and the actual role of silicon dioxide. It is planned to carry out animal experiments with a shorter running time.

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