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Abstract

The easy clinical handling and applicability of biomaterials has become a focus of materials research due to rapidly increasing time and cost pressures in the public health sector. The present study assesses the in vitro and in vivo performance of a flexible, mouldable, cottonwool-like nanocomposite based on poly(lactide-co-glycolide) and amorphous tricalcium phosphate nanoparticles (PLGA/TCP 60:40). Immersion in simulated body fluid showed exceptional in vitro bioactivity for TCP-containing fibres (mass gain: 18%, 2 days, HAp deposition). Bone regeneration was quantitatively investigated by creating four circular non-critical-size calvarial defects in New Zealand White rabbits. The defects were filled with the easy applicable cottonwool-like PLGA/TCP fibres or PLGA alone. Porous bovine-derived mineral (Bio-Oss) was used as a positive control and cavities left empty served as a negative control. The area fraction of newly formed bone (4 weeks implantation) was significantly increased for TCP-containing fibres compared to pure PLGA (histological and micro-computed tomographic analysis). A spongiosa-like structure of the newly formed bone tissue was observed for PLGA/TCP nanocomposites, whereas Bio-Oss-treated defects afforded a solid cortical bone.
In vivo and in vitro evaluation of flexible, cotton wool like nanocomposites as bone substitute material for complex defects

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Abstract

Easy clinical handling and applicability of biomaterials has shifted into the focus of material’s research through the rapidly increasing time and cost pressure in the public health sector. The present study assesses the in vitro and in vivo performance of a flexible, moldable, cotton-wool like nanocomposite based on poly(lactide-co-glycolide) and amorphous tricalcium phosphate nanoparticles (PLGA/TCP 60:40). Immersion in simulated body fluid showed exceptional in vitro bioactivity for TCP containing fibres (mass gain: 18%, 2 d, HAp deposition). Bone regeneration was quantitatively investigated by creating four circular non critical-size calvarial defects in New Zealand White rabbits. The defects were filled with the easy applicable cotton wool-like PLGA/TCP fibres or PLGA alone. Porous bovine-derived mineral (Bio-Oss®) was used as a positive control and cavities left empty served as a negative control. The area fraction of newly formed bone (4 weeks implantation) was significantly increased for TCP containing fibres if compared to pure PLGA (histological and micro computed tomography analysis). A spongiosa-like structure of the newly formed bone tissue was observed for PLGA/TCP nanocomposites whereas Bio-Oss® treated defects afforded a solid cortical bone.

Keywords

Bone regeneration, nano calcium phosphate, PLGA, electrospinning, rabbits
1. Introduction

The use of bone substitute material for non-load bearing operation sites has recently experienced increased interest. Such materials find application in oral and maxillofacial surgery. Two properties determine the success of a bone graft: Its handling properties in the operation room and its performance in vivo i.e. formation of new bone. An easy handling generally safes time and minimizes complications during surgery and therefore reduces cost. An uncomplicated, fast post-operative healing means less stress and less cost for the patient. Autogeneous bone [1, 2] (taken from iliac crest, ribs or calvarium) is frequently used for the repair of bone defects. However, besides of its limited availability, its use often results in significant donor site morbidity [3], requirement of a second surgical site, potential risk of viral or bacterial infections [4] and of an immune response of the host tissue after implantation. Therefore, artificial bone substitutes are being developed [5-11]. Preferably, these materials are bioactive for spontaneous bone bonding in the living body, otherwise the implant may be surrounded by fibrous tissue and encapsulated, in the worst case leading to device failure and explantation. Additionally the bone substitute should be resorbed and gradually replaced by newly formed bone [12].

Different types of bioactive materials, mainly ceramics, have been developed over the last years and are clinically used in the form of hydroxyapatite (HAp), tricalcium phosphate (TCP), HAp/TCP bi-phase ceramics and bioactive glass. Calcium phosphates are osteoconductive [13, 14] and support the attachment, differentiation and proliferation of osteoblasts as investigated in vitro and in vivo. Additionally they have an excellent biocompatibility due to their close chemical and crystal similarity to natural bone mineral [15]. The greatest limitation of pure ceramic biomaterials is their
brittleness, incompressibility and difficulty to fill irregularly shaped bone defects during surgery.

The incorporation of ceramics in biodegradable and bioresorbable polymers has been investigated previously to improve the ductility and mechanical properties of the biomaterial [16]. Poly(lactide-co-glycolide) (PLGA) is a well established FDA (Food and Drug Administration) approved [17] biodegradable synthetic copolymer for scaffolds in tissue engineering [18] that allows resorption over time and regeneration of biological tissue. Suitable 3D scaffolds require an interconnected porosity of > 90% to direct the cells in tissue growth and vascularization [19, 20]. Electrospinning [21] is a simple process that offers ultrafine polymer fibres having a high specific surface area. Modified to low-temperature electrospinning [22] this method enables access to scaffolds with high porosities.

The beneficial use of ceramics in a nanoparticulate form as fillers in biopolymers has been demonstrated in numerous investigations: Misra et al. have compared nanoscale and microscale bioactive glass in poly(3-hydroxybutyrate) films prepared by solvent casting and showed increased in vitro bioactivity, mechanical properties and protein adsorption for the nanocomposite [23]. Nanofibrous electrospun scaffolds consisting of biodegradable polycaprolactone, hydroxyapatite and natural polymer gelatine provided a large surface area for cell attachment, proliferation and mineralization [24]. Amorphous TCP has been shown to exhibit high in vitro bioactivity [25, 26] and increased solubility and reactivity attributed to its high enthalpy of formation [27]. In a recent study [28, 29] an electrospun nanocomposite containing amorphous TCP exhibited a high in vitro bioactivity, cell proliferation and osteogenic differentiation of human mesenchymal stem cells.
The present study investigates a combination of these advantageous material properties in the form of a cotton wool-like, amorphous TCP containing PLGA scaffold prepared by low-temperature electrospinning. The potential of this bone substitute for clinical application in repairing non-load bearing bone defects as they occur in dental, oral and maxillofacial surgery was tested. *In vivo* bone regeneration was investigated by creating four circular non critical-size calvarial defects in New Zealand White rabbits [30] which were filled with the easy to apply electrospun PLGA/TCP fibres or PLGA fibres alone. As the “gold standard” in bone substitution, a porous bovine-derived mineral (Bio-Oss®) was used as a positive control, cavities left empty served as a negative control. Histology and micro computed tomography were performed to evaluate the formation of new bone. The goal of the present study was to show the first *in vivo* performance of a flexible, moldable and highly bioactive calcium phosphate/PLGA nanocomposite.
2. Materials and Methods

2.1. Preparation of TCP particles

X-ray amorphous tricalcium phosphate nanoparticles (TCP, chemical formula: Ca₃(PO₄)₂) were prepared by flame spray synthesis using calcium hydroxide (Riedel de Haen, Ph. Eur.) dissolved in 2-ethylhexanoic acid (Fluka, puriss) and tributyl phosphate (Fluka, puriss) as precursors [31-33]. The liquid mixture with a molar ratio of Ca/P = 1.5 was diluted with toluene (Fluka, puriss) (2:1 vol/vol) and fed through a capillary (diameter 0.4 mm) into a methane/oxygen flame at a rate of 5 ml min⁻¹. Oxygen (5 L min⁻¹, 99.8%, Pan Gas) was used to disperse the liquid leaving the capillary and resulted in a burning spray of about 10 cm height. The as-formed particles (production rate: 8 g h⁻¹) were collected on a teflon filter (25.7 cm diameter, Tulona needle felt-PTFE-membrane), placed on a cylinder mounted above the flame, by the aid of a vacuum pump (Busch Seco SV 1040 C). As a gold standard for bone regeneration bovine bone mineral (Bio-Oss®) was donated by Geistlich (Wohlhusen, Switzerland).

2.2. Particle characterization

The specific surface area (SSA) of the TCP powder was measured by nitrogen adsorption at 77 K according to the Brunauer-Emmett-Teller (BET) method after outgassing at 150 °C for 1h. The primary particle diameter was calculated according to \( d_{\text{BET}} = \frac{6}{(\rho_{\text{TCP}} \cdot \text{SSA})} \) assuming spherical particles, whereas the density of TCP equals 3.14 g cm⁻³. For Fourier transform infrared (FTIR) spectroscopy, 1% (w/w) of powder was mixed with KBr (Fluka, puriss) and examined on a Tensor 27 spectrometer (Bruker Optics, 4000 cm⁻¹ < \( \lambda \) < 400 cm⁻¹, 16 scans, 4 cm⁻¹ resolution) equipped with a diffusive reflectance accessory (DiffusIR™, Pike Technologies). X-
ray diffraction (XRD) patterns were collected on a Stoe STADIP2 (Ge monochromator, Cu Kα1, PSD detector). Transmission electron microscopy (TEM) images were recorded on a CM30 ST (Philips, LaB6 cathode, operated at 300 kV, point resolution ~4 Å). Particles were deposited onto a carbon foil supported on a copper grid.

2.3. Scaffold preparation

Clinically approved poly(lactide-co-glycolide) (PLGA) with a copolymer ratio of 85:15 (Resomer® Sample MD Type RG) was purchased from Boehringer Ingelheim with a weight and number average molecular weight of 380’300 g mol⁻¹ and 181’900 g mol⁻¹, respectively. Randomly oriented pure PLGA and 40 wt% TCP containing PLGA fibres were fabricated by low-temperature electrospinning [22], a process that is slightly modified from common electrospinning [21, 34] and enables to produce scaffolds with high porosities. Both electrospinning solutions were prepared with a concentration of 7% (w/w) PLGA in chloroform (Riedel de Haen, Ph. Eur.) containing 5% (w/w) of the surfactant Tween20 (Polysorbate20, Fluka, Ph. Eur.) referred to the polymer. For the preparation of the electrospinning solution, TCP nanoparticles were first dispersed in a chloroform/Tween20 mixture using an ultrasonic processor at 320 W for 7 min applying pulsed intervals to allow for relaxation of the particles. PLGA was subsequently added and dissolved for 15 h by magnetic stirring. The electrospinning solutions were fed through a capillary (inner diameter 1.0 mm) using a syringe pump. The feeding rate was set to 2 ml h⁻¹ for PLGA/TCP 60/40 and 4 ml h⁻¹ for the pure PLGA solutions. A high voltage supply was used to apply voltages of 20 kV to the needle tip. A coaxial jacket of chloroform saturated air prevented the needle exit form clogging by suppressing excessive solvent
evaporation as proposed by Larsen et al. [35]. A positively charged jet was formed from the Taylor cone and was sprayed onto a rotating (130 rpm) collection drum covered by aluminium foil. The drum was loaded with dry ice (frozen CO₂; average temperature 195 K) which caused rapid deposition of ice crystals at the cold external cylinder surface on which the fibres were collected. The distance between the needle tip and the collection tube (diameter 8 cm) was kept at 10 cm for PLGA/TCP 60/40 and 20 cm for pure PLGA samples. Immediately after electrospinning the as-spun scaffolds were transferred to a vacuum oven (50 mbar, RT, 12 h).

2.4. Scaffold characterization
The morphology of the electrospun fibres was characterized using scanning electron microscopy (SEM; LEO 1530 Gemini) at a voltage of 3 kV after sputtering the samples with a 4 nm platinum layer. The porosities of the scaffolds were calculated according to \( \varepsilon = \frac{V_{\text{tot}} - V_{\text{solid}}}{V_{\text{tot}}} \), whereas the total volume \( V_{\text{tot}} \) was determined from the base area of the sample and its thickness as measured by SEM. The solid volume \( V_{\text{solid}} \) is given by the sample mass and its bulk density calculated from the density of PLGA \( \rho_{\text{PLGA}} = 1.2 \text{ g cm}^{-3} \) and TCP \( \rho_{\text{TCP}} = 3.14 \text{ g cm}^{-3} \).

2.5. In vitro biomineralization in simulated body fluid
Electrospun PLGA and TCP containing nanocomposite scaffolds were cut into rectangles (70 × 10 mm²) for in vitro degradation tests. After sterilization by UV irradiation (50W m⁻², 2h) [36], the samples were placed in 250 ml simulated body fluid (SBF, pH 7.4) and incubated for 49 h at 37°C. SBF was prepared according to Kokubo et al. [37] and sterile-filtered through a presterilized filter unit (Millipore, 0.22μm). Sterile conditions guaranteed that no bacterial or fungal degradation resulted
in polymer dissolution. After immersion in SBF, the samples were washed with Millipore water, dried for 24 h in an evacuated exsiccator at room temperature before SEM images were recorded. A more detailed study on the in vitro biomineralization of these nanocomposites may be found in Ref [28].

2.6. In vivo study in New Zealand white rabbits

For the in vivo study PLGA and PLGA/TCP scaffolds were portioned and UV-sterilized (50W m$^{-2}$, 2h). Nine New Zealand white rabbits were used for the study, which was approved by the local Ethical Committee. The rabbits were sedated by Ketamin and further anaesthetized by a Halothan-N$_2$O inhalation method. The surgical area was clipped and prepared with iodine for aseptic surgery. A linear incision was made from the nasal bone to the midsagital crest. The soft tissues were reflected and the periosteum was dissected from the site (occipital, frontal, and parietal bones). Four 6 mm craniotomy defect were created (2 in the parietal and 2 in the frontal bone) with a 6 mm trephine in a dental hand piece. The surgical area was flushed with saline act as a coolant and to remove bone debris. To avoid any dural perforation, the defects marked half way through the bone by the trephine were finally made by a round blur creating a 6 mm defect. In each animal, one untreated defect was compared to one defect treated with Bio-Oss$^\text{®}$ (Geistlich, Wolhusen, Switzerland), one treated with PLGA and one treated with PLGA/TCP 60:40 scaffolds. After placement of the materials, the soft tissues were closed with sutures. Analgesia was provided by injection of Novalgin (50 mg/kg). Four weeks following the surgery, the rabbits were sacrificed to harvest the calvarial bone after sedation with barbiturates by an overdose of Ketamin.
2.7. Radiography and histology

Specimens were radiographed using a dental radiography unit with ultra speed dental films (Eastman Kodak Company, NY, USA). The radiographs were photographed, scanned and later used to localize the middle section of the defects. After radiography, the samples were first prepared with a sequential water substitution process that involved 48 h in 40% ethanol, 72 h in 70% ethanol (changed 24 h). At this step the µCT scan was performed. Following µCT the sequential water substitution was continued by placing the samples 72 h in 96% ethanol and finally 72 h in 100% ethanol. Following the water substitution, samples were placed in xylene for 72 h for defatting the recovered bone (changed every 24 h). Next, infiltration was performed by placing the samples in methyl methacrylate (MMA) for 72 h (Fluka, purum) followed by three days in 100 ml MMA + 2 g di-benzoylperoxide (Fluka, purum), at 4 °C. Samples were embedded by placing them in 100 ml MMA + 3 g di-benzoylperoxide + 10 ml plastoid N or dibutylphtalate (Merck, for synthesis) and allowing polymerization to occur at 37 °C in an air tight water bath. 4.5 µm sections were prepared from the midst of the defects and stained with Goldner Trichrome. Digital images were taken with an image analysis program (Adobe Photoshop).

2.8. Histomorphometry

Quantitative evaluation of bone regeneration was assessed in the middle section by determining the percentage of bone within the former defect area. Measurements were carried out directly in the digital images at a magnification of 12.5. The digital pictures were taken with a superimposed scale in order to help to identify the defect margins metrically. Using the calvarial bone thickness at the borders of the 6mm defects, the total area of the defect was identified. Thereafter, the number of pixels
within the total defect area was counted using commercial software (Adobes Photoshop CS). The borders of the mineralized bone within the former defects area were manually marked on the computer screen using a digital pen. Subsequently, the pixels within this marked area were counted by the software. The area fraction of bone was calculated as follows:

\[
\text{Area fraction of newly formed bone (\%) = } \frac{\text{Pixel number of the bone area}}{\text{Total pixel number of defect}} \cdot 100
\]

2.9. Micro-computed tomography (µCT)

Before embedment in methacrylate, the intact specimens stored in 70% EtOH were subjected to µCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) as described previously [38]. In brief, µCT was performed using an isotropic resolution of 30mm. A constrained Gaussian filter (\(\sigma = 1.2\) and width = 1) was used to suppress the noise in the volumes. Mineralized bone tissue was distinguished from non-mineralized tissue and graft material using two global thresholds of 19% and 30% of the maximum possible gray-scale value, respectively. A one-voxel thick dilation was then applied to the graft material, to remove partial volume elements resulting from digitization and filtration. At the initial defect location, a cylindrical volume of interest (VOI) was identified for quantitative analyses, allowing for a three-dimensional assessment of bone volume and graft as described in Jung et al [30].

2.10. Statistical Analysis

Mean values and standard deviation were calculated for the amount of bone formation within the former defect area or the former defect volume. Significant differences were identified by repeated measures ANOVA using the post hoc Fisher LSD (Least Significant Difference) test. The paired two-tailed t-test was used to compare
individual groups with each other. Statistical significance was set at $\alpha<0.05$. Values were displayed as box-plots ranging from the 25th (lower quartile) to the 75th (upper quartile) percentile including the mean and whiskers showing the minimum and maximum values. Statistical analysis was performed by using a statistical software package (SPSS 12.0 for Windows).
3. Results

3.1. TCP nanoparticles

Spherical and highly agglomerated amorphous TCP nanoparticles in the size range of 20-50 nm were successfully produced by flame spray synthesis as shown in earlier studies [31]. The as-prepared TCP particles exhibited a specific surface area of $85 \pm 2 \text{ m}^2 \text{ g}^{-1}$ resulting in a volume averaged sphere equivalent particle diameter $d_{\text{BET}}$ of 22 nm. The high phase purity of the nano-powder was confirmed by applying Fourier-transform infrared spectroscopy (see supplementary information). Distinct, characteristic peaks for $\beta$-TCP were obtained after sintering at 900°C while peaks for impurities such as pyrophosphate (indicative of an excess of phosphate) and hydroxyapatite (indicative of an excess of calcium) were absent [31]. X-ray diffraction patterns of TCP showed a broad peak at a $2\Theta$ value of 31.0° characteristic for amorphous TCP and the corresponding crystalline phase of $\beta$-TCP after sintering (see supplementary information).

3.2. PLGA/TCP Scaffolds

Pure and 40 wt% TCP containing PLGA scaffolds were prepared by low-temperature electrospinning. The three dimensional meshes consisted of homogeneous fibres within a diameter range of 5-10 $\mu$m as shown in the SEM images (Fig. 1). A high mesh porosity of 95% was achieved which is consistent with an earlier study on low-temperature electrospinning [22]. The fibres itself exhibited a porous structure and the surface of TCP particle containing fibres clearly revealed an increased roughness and surface area when compared to pure PLGA fibres (Fig. 1b, d).

The scaffolds could be manually processed into a cotton wool-like nanocomposite allowing easy proportioning and handling as well as an advantageous adaptation of a
bone defect. The flexible and compressible properties of this implant material are demonstrated in Fig. 2. It was shown that the biomaterial expands up to 35 % of its compressed volume. The packing density and the TCP amount in the composite are variable and can be adjusted depending on the defect geometry and surgical needs. Misplacement during operation can be corrected by easy removal of the entire scaffold while granular bone substitutes are more complex to remove.

3.3. In vitro bioactivity

Previous to starting the in vivo study in rabbits the in vitro bioactivity of the cotton-wool like nanocomposite was investigated. The change in the surface morphology during immersion in simulated body fluid was analyzed using scanning electron microscopy (SEM). After 49 hours immersion, the surface of pure polymer fibres became rougher (Fig. 3a) and small holes were observed due to the dissolution of the water soluble surfactant Tween20 and the degradation of the PLGA. In contrast, a continuous, nano-featured and ~1μm thick hydroxyapatite layer was deposited on the surface of TCP containing fibres (Fig. 3b, c) which stays in line with a previous in vitro study [28] on electrospun PLGA/TCP nanocomposites. An important aspect in developing a bone substitute material is the rate at which calcium phosphate mineral formation occurs under physiological ion concentration. Therefore, we investigated the change in mass caused by mineral deposition on the scaffolds. After 48 hours immersion time in simulated body fluid the mass of pure PLGA fibres decreased by 1% most likely due to the dissolution of the water soluble surfactant Tween20. On the other hand, the mass of TCP containing fibres increased by 18 % within the same time period. This change in mass can be mainly attributed to the strongly enhanced formation of hydroxyapatite on the surface of amorphous TCP
containing composites. These findings are in agreement with the results from a previous study [28] and confirm the very high in vitro bioactivity of the flexible biomaterial. Further it reflects the high reactivity of amorphous TCP nanoparticles [27, 39].

3.4. Surgical procedure

New Zealand white rabbits were chosen for the in vivo study because they are larger animals than mice or rats and have a better comparability to humans. Bone regeneration was evaluated in four circular non-critical size defects (Ø = 6mm) in the cranial of totally 9 rabbits. In each animal the four defects were treated with pure PLGA and the highly in vitro bioactive PLGA/TCP 60:40 scaffolds and compared to Bio-Oss® and an untreated defect (Fig. 4). Bio-Oss® is a commercial available natural and osteoconductive bone substitute that is applied in dental surgery as the current gold standard. It consists of the mineral portion of bovine bone. From the surgical point of view the handling of the electrospun scaffolds was easy (application like stuffing cotton wool into a defect geometry; see Figure 2) and compared to Bio-Oss® the packing density (degree of compaction when filling the defect; Figure 2) and mineral content in the composite material (flexibility during material preparation) can be adjusted depending on the defect geometry and surgical needs. Both PLGA and PLGA/TCP scaffolds were easily wetted with blood (Fig. 4b) favoring a homogeneous distribution of cells and nutrients within the bone substitute. The granular Bio-Oss® reference material was more challenging to apply and fix in the bony defect (some granules could be found outside the defect, Fig. 4b defect 3) as the small grains are often washed around the operation site during wound cleaning using physiological saline. For the treatment with granular bone substitute an average mass
of 35 mg per defect were used while only 10 mg and 16 mg of foreign material had to be applied for PLGA and PLGA/TCP respectively.

3.5. Descriptive histology of the in vivo study in rabbits

Microscopic examination of the treated cranial defects demonstrated new bone formation mainly originating from the bony borders directed toward the centre. Complete bridging of the former defects with mineralized bone occurred in 2 of the PLGA/TCP samples (Fig. 8a, b). Solid cortical bone developed around the Bio-Oss® particles (Fig. 5b, 6a, d) with a large ratio of original implant material remaining in the site. For the here introduced flexible materials, the newly formed bone showed a more spongy (spongiosa-like) appearance in both the PLGA (Fig. 5c, 6b, e) and PLGA/TCP group (Fig. 5d, 6c, f). The solid appearance of the newly formed bone covering the Bio-Oss® particles was also evident in the Bio-Oss® group at higher magnifications (Fig. 6d). In the PLGA group small round holes of 3.5-5.0 µm in diameter were localized in the newly formed bone tissue (Fig. 6e). The same small holes were present in the PLGA/TCP group, but at a lower rate. In addition to the small round holes newly formed bone in the PLGA/TCP group contained also bigger holes of round to more irregular shape of 8.0 - 12.0 µm diameter. The cell density in the non-mineralized portion appeared to be increased in the PLGA/TCP group compared to the PLGA group and more resembling the situation of the non mineralized tissue of the Bio-Oss® group (Fig. 6). No cellular infiltrate such as macrophages could be seen in any of the treatment groups.
3.6. Quantitative histomorphometric analysis

The average area fractions of newly formed bone measured within the former defect amounted to 28.4±14.9% for the empty defect, 30.8±14.3% for Bio-Oss® treated defects, 25.1±14.6% for the PLGA treated defects, and 34.9±17.0% for the PLGA/TCP treated defects (Fig. 7). By using ANOVA LSD no significant difference between the 4 groups could be detected. Since all animals were treated with all 4 treatment modalities, we apply the paired t-test to find a significant (p=0.07) higher area fraction of newly formed bone in the PLGA/TCP group compared to the PLGA group. Thus, the addition of the TCP nanoparticles to the electrospun PLGA improved its in vivo performance as bone regeneration scaffold.

3.7. Micro-computed tomography (μCT)

μCT was performed of the entire calvarial bone as shown in figure 8a, b. Pair wise comparison of PLGA and PLGA/TCP treated defects show that in all cases the defect is filled by more hard tissue in the PLGA/TCP group than in the PLGA group respectively (Fig 8c). In agreement with the results obtained by histomorphometry, the μCT showed higher values for bone volume in the PLGA/TCP than in the PLGA only group, although paired t-test failed to show significance (p=0.167). The μCT-derived values were 8.42±4.35 mm³ for the untreated control group, 12.31±4.57 mm³ for the Bio-Oss® group, 5.18±3.60 mm³ for the PLGA group, and 8.13±4.24 mm³ for the PLGA/TCP group. Since substantial amounts of bovine bone mineral composed Bio-Oss® were still present at 4 weeks (Fig 5b, 6d), the X-ray attenuation of the graft material was very similar to that of bone tissue. Therefore the segmentation of the μCT signal using global thresholds to distinguish Bio-Oss® from new bone was
almost impossible and could lead to an overestimation of the bone volume in the Bio-Oss® treated control samples.

4. Discussion

The demand for flexible and easy to apply implant material for the repair of complex shaped bone defects increases. In this study we combined favorable properties such as ductility/flexibility of polymer fibers with the stability and bioactivity of the calcium phosphate ceramic. The performance of a flexible and cotton-wool like material as bone substitute was investigated in vivo in New Zealand white rabbits and compared with the today’s gold standard in dental surgery (Bio-Oss®) [40].

The investigated nanocomposite exhibits high bioactivity and meets surgical handling requirements by combining positive features of flame derived amorphous tricalcium phosphate nanoparticles and biodegradable PLGA by fiber processing through electrospinning. Prior to the in vivo study in rabbits, the in vitro bioactivity of the biomaterial was investigated and stayed in line with earlier studies on amorphous TCP [27] and PLGA/TCP nanocomposites [28, 39]. In vitro degradation in simulated body fluid showed an exceptional high degree of biomineralization for TCP containing samples (mass increase 18% within two days) suggesting a fast bone regeneration. During the implantation time, the investigated materials were observed to be fully compatible with the surrounding tissue without showing any significant adverse reactions. No inflammatory response was determined disclosing the biocompatibility of the used materials. Remarkable was the better wettability of the PLGA/TCP composite in comparison to the PLGA only material, since it could increase the stabilization of the blood clot needed for optimal bone regeneration [41].

Four weeks after implantation, descriptive histology illustrated that the Bio-Oss®
particles were surrounded by solid cortical bone. The large remaining of non-degraded Bio-Oss® particles might act as a barrier to new bone formation and leads to an overestimation of regenerated bone in the μCT analysis. It is known that Bio-Oss® needs several years of implantation before it shows some slow in vivo resorption [42].

The newly formed bone in the defects treated with PLGA and PLGA/TCP nanocomposites had a spongeous structure. At this stage of bone formation the defects might lack mechanical stability, comparable to Bio-Oss® treated defects. However, the need for early stability in non-load bearing sites is of minor importance. The electrospun samples are not fully degraded after 4 weeks implantation (Fig. 6e and f) but an increased surface is noticeable.

The incorporation of TCP nanoparticles in PLGA fibres significantly increased (p = 0.07) the average area fraction of newly formed bone when compared to pure PLGA fibers (Fig. 7). This finding is in agreement with μCT results (Fig. 8).

Based on results from the in vitro study in simulated body fluid a more pronounced difference in bone regeneration between the PLGA and PLGA/TCP nanocomposites was expected. This clearly demonstrates the importance of carrying out bioactivity tests besides in vitro additionally in vivo in order to get complete information about the performance of the bone substitute. Before first human trials, the nanocomposites would have to undergo further investigations in animals to evaluate the long term stability and clinical outcome.
5. Conclusions

The advantageous use of a flexible and cotton-wool like PLGA/TCP nanocomposite for bone regeneration was investigated in nine New Zealand white rabbits by applying a circular defect model (Ø = 6 mm) in the cranium. The implant material combined a high *in vitro* bioactivity, surgical handling requirements and suggests application in non-load bearing, complex shaped bone defects. The area fraction of newly formed bone after four weeks implantation time was significantly increased in amorphous TCP containing PLGA fibres when compared to neat polymer electrospun scaffolds. The newly formed bone of these nanocomposite treated defects had a spongiosa bone like appearance whereas a solid cortical bone was developed in defects treated with Bio-Oss®, the today’s gold standard in dental surgery. A direct comparison between bone regeneration of treated defects with PLGA/TCP nanocomposites and Bio-Oss® using µCT was hindered since Bio-Oss® consists of bovine bone mineral which was difficult to distinguish from newly formed bone.

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References

22. Simonet M, Schneider OD, Neuenschwander P, Stark WJ. Ultraporous 3D polymer meshes by low-temperature electrospinning: Use of ice crystals as a


Figures

Fig. 1. Scanning electron microscopy images of as electrospun scaffolds: Overview (left) and close up (right) of pure PLGA (a, b) and PLGA/TCP 60/40 fibres (c,d). The high surface structuring is related from the use of a biodegradable surfactant as a pore template.

Fig. 2. Bone graft substitute materials used for the in vivo study. (a) Electrospun pure PLGA and PLGA/TCP scaffolds can be uncompressed in a cotton wool like appearance. (b) Simulated compressibility of the flexible scaffold and expansion afterwards up to 35%. (c) Bio-Oss® granules used as a today’s gold standard for bone regeneration.
Fig. 3. Scanning electron microscopy images after 2 days in vitro biomineralization in SBF: Cross section of a (a) pure PLGA fibre and (b) PLGA/TCP 60/40 fibre with a HAp layer of \( \approx 1 \mu m \). (c) The deposition of nanostructured hydroxyapatite occurred only for TCP containing PLGA fibres.

Fig. 4. Surgical procedure. (a, b) Filling of the cranial defects (Ø 6 mm) in New Zealand White rabbits: Pure PLGA (1), PLGA/TCP nanocomposite (2), Bio-Oss\textsuperscript{®} (3), empty as a negative control (4). The electrospun scaffolds can be easily applied. TCP containing PLGA fibres leading to an increased wetability soak up the surrounding body fluid faster compared to pure PLGA fibers.
Fig. 5. Histological sections of the cranial defects in rabbits after 4 weeks of healing: (a) No filling as a negative control, (b) Bio-Oss® as a gold standard, (c) pure PLGA and (d) PLGA/TCP nanocomposite; scale in millimeters.

Fig. 6: Histological sections of the cranial defects in higher magnifications: (a, d) Bio-Oss®, (b, e) pure PLGA and (c, f) PLGA/TCP nanocomposite; scales provided are 50 µm. The area shown in the higher magnification is indicated. The new formed bone in the electrospun fibrous PLGA and PLGA/TCP nanocomposite has a finer, more spongy appearance compared to the more solid cortical bone formation with Bio-Oss®.
Fig. 7. Analysis of bone formation. Area fraction of newly formed bone within the former defect in percent of the total defect area. Histomorphometrical results (box plot with median and whiskers).

Fig. 8. Micro-computed tomography. (a, b) Two examples of the μCT of the entire cranial bone are shown. Defect margins and treatment modalities are indicated. (c) μCT based direct intra-animal comparison of PLGA and PLGA/TCP treated defects of all nine animals. In all animals with the exception of one the closure of the PLGA/TCP treated defects is advanced compared to the PLGA treated defect in the same animal.
Supplementary Figures:

Fig. 1: Characterization of flame spray synthesized nanoparticles: (a) Transmission electron micrograph of as-prepared tricalcium phosphate (TCP) nanopowder (b) Fourier-transform infrared spectra of as-prepared TCP nanoparticles and distinct peaks characteristic for $\beta$-TCP after sintering. The absence of impurities such as apatite ($\sim$3600 cm$^{-1}$) and calcium pyrophosphate ($\sim$720 cm$^{-1}$) confirm the purity of the material. (c) Broad XRD pattern of as-prepared amorphous TCP nanoparticles and peaks characteristic for $\beta$-TCP after sintering at 900°C.