

Monitoring Degradation Process of PLGA/CaP Scaffolds Seeded With Mesenchymal Stem Cells in a Critical-Sized Defect in the Rabbit Femur using Raman Spectroscopy

Beltzer C¹, Hägele J², Kratz M³, Fuhrmann R⁴, Wilke A⁵, Franke RP⁴ and Endres S^{6*}

¹Department of General Surgery, Ulm Military Hospital of German Armed Forces, Ulm, Germany

²Department of Radiology, University Hospital of Schleswig-Holstein, Lübeck, Germany

³Department of Experimental Orthopaedics, University Hospital Giessen and Marburg, Marburg, Germany

⁴Central Institute for Biomedical Engineering, University of Ulm, Ulm, Germany

⁵Department of Orthopaedics, Elisabeth Hospital GmbH, Olsberg, Germany

⁶Department of Orthopaedics, Lörrach Hospitals GmbH, Rheinfelden, Germany

Abstract

Bone Tissue Engineering (BTE) and the use of Bone Replacement Materials (BRM) has become a growing field of research during the last decades due to the emergence of regenerative medicine. However, ideal BRM is still not available for the needs of clinical practice due to several limitations of material properties. Biodegradable polyester poly(lactide-co-glycolide)/calcium phosphate (PLGA/CaP) is very popular among BRM and often used for BTE applications. Fast material shrinkage of PLGA scaffolds *in vivo* with loss of its function as a guiding structure for in growing blood vessels and as an osteoconductive construct has been described in the past. In order to receive new information about the *in vivo* degradation process of these PLGA/CaP scaffolds, Raman Spectroscopy was included in this study to provide further scaffold material analysis at different points of time: PLGA/CaP scaffolds (seeded with or without mesenchymal stem cells, MSCs) were implanted in a 12.0 mm Critical-Sized Defect (CSD) of the rabbit femur. Animals were sacrificed after 4 and 26 weeks. Samples were then investigated using Micro-computer tomography (μ -CT) and histology. Samples of two animals previously examined were then investigated using Raman Spectroscopy. In fact, the use of Raman Spectroscopy generated new knowledge about the different steps of degradation and material behavior of PLGA/CaP scaffolds *in vivo*: CaP embedded in and coated on the scaffold was dissolved completely after 4 weeks, and maintenance of the scaffold's structure and interconnectivity was provided by PLGA alone. Therefore Raman Spectroscopy must be considered as a valuable tool for characterization of BRM and bone tissue in the field of BTE in the future.

Keywords: Bone tissue engineering; PLGA; Scaffold; Degradation; Raman spectroscopy; Mesenchymal stem cells

Introduction

Material properties, design and architecture of Bone Replacement Materials (BRM), such as poly (lactide-co-glycolide)/calcium phosphate (PLGA/CaP) synthetic scaffolds, affect biocompatibility as well as their osteoconductive and osteoinductive effects and potential [1]. Mechanical characteristics and material properties of BRM has a great impact for the successful application in a Critical Sized Defect (CSD) of bone [2,3]. Particularly osteoinductivity has been illustrated with CaP coated BRM [4]. Tissue engineering using mesenchymal stem cells (MSCs) leads to superior healing results versus tissue engineering without MSCs [2,5].

However, fast *in vivo* degradation of PLGA scaffolds with loss of its origin shape and interconnectivity is common after implantation [6,7], shrinking can also be observed *in vitro* [1]. It was found that degradation of PLGA 75/25 was faster *in vivo* than *in vitro* [8]. Due to the fast degradation BRM can lose its function as a guiding structure for migrating cells and in growing blood vessels [6]. This can lead to a lack of blood supply and nutrition in the central scaffold volume with subsequent absent or scarce development of new bone tissue in the central region [7]. Therefore, fast degradation of a BRM should not occur after implantation in a CSD. It is common that degradation is influenced by a lot of factors including material composition, temperature, pH as well as by the scaffold's architecture such as pore size and porosity [1]. There is minimal data available regarding the process of *in vivo* degradation of BRM: "So, although plenty of reports concern degradation of PLGA in various forms such as films and microsphere, the investigation of degradation of porous scaffolds is still valuable in its own right [1]."

For most studies X-ray -, μ -CT- and histological examinations are used to assess biocompatibility of the implanted BRM in a CSD. Results of μ -CT and histological examinations of this study have been previously published in more detail [7]. However, these examinations do not provide any information about the qualitative steps and chemical changes of BRM during the degradation process. This information would be valuable to improve scaffold processing techniques in order to modify mechanical properties of BRM with the intention to avoid fast degradation and shrinkage of applications in the future.

The aim of this study was to evaluate the use of Raman Spectroscopy in order to achieve more information about the qualitative and chemical degradation progress, mineral and matrix changes of PLGA/CaP scaffolds following implantation in a rabbit's femur at different points of times. Mechanical loading of scaffolds with calcium has been described to accelerate the PLGA degradation [9].

Raman Spectroscopy is in common use in the investigation

***Corresponding authors:** Stephan Endres, Department of Orthopaedics, Lörrach Hospitals GmbH, Rheinfelden, Germany, Tel: +49 7621 4160; E-mail: Endres.Stefan@klinloe.de

Received October 5, 2013; **Accepted** October 31, 2013; **Published** November 7, 2013

Citation: Beltzer C, Hägele J, Kratz M, Fuhrmann R, Wilke A et al. (2013) Monitoring Degradation Process of PLGA/CaP Scaffolds Seeded With Mesenchymal Stem Cells in a Critical-Sized Defect in the Rabbit Femur using Raman Spectroscopy. J Bone Marrow Res 1: 133. doi: [10.4172/2329-8820.1000133](https://doi.org/10.4172/2329-8820.1000133)

Copyright: © 2013 Beltzer C, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

of molecular structures and has already been used to specify calciumphosphate(CaP)-coated polymers and BRM [10-12]. It was also used for *in vivo* examinations in an animal model [13]. This spectroscopic device makes it possible to differentiate between organic and mineral elements as well as obtain accurate information about the chemical composition of materials (Figure 1 and 2).

The advantages for the application of Raman Spectroscopy are described by Tarnowski: "Specimen preparation is minimal, and tissues that have been fixed, processed, and embedded can be examined, and intact specimens that have not been fixed, processed or embedded can also be examined."

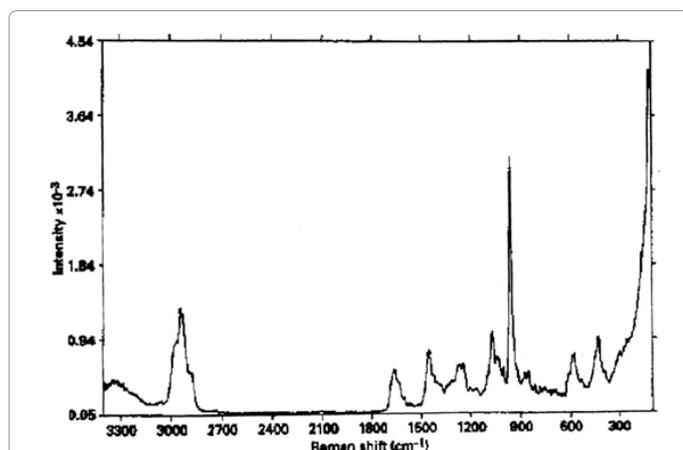


Figure 1: Raman spectrum of whole human bone [20]. The spike with the highest intensity can be observed at a wave length of 960 rel. 1/cm, detecting phosphate as a main element of hydroxyapatite. Therefore it is interpreted as an indicator of mineralized bone tissue [12].

Assignment	Wave number range (cm ⁻¹)	Observed in
PO ₄ ³⁻ ν ₂	422-454	Raman
PO ₄ ³⁻ ν ₄	578-617	Raman/IR
C-C stretching	815-921	Raman
CO ₃ ²⁻ ν ₂	860-890	IR
PO ₄ ³⁻ ν ₁	957-962	Raman
HPO ₄ ²⁻ ν ₃	1003-1005	Raman
PO ₄ ³⁻ ν ₃	1006-1055	Raman
CO ₃ ²⁻ ν ₁	1065-1071	Raman
Amide III	1243-1269	Raman
CH ₂ wag	1447-1452	Raman/IR
Amide II	1540-1580	IR
Amide I	1595-1720	Raman/IR
CH ₂ stretching	2840-2986	Raman

Figure 2: Characteristic Raman bands in bone tissue wave number ranges and their corresponding chemical assignments.

Materials and Methods

Scaffold processing

Macroporouspoly(lactide-co-glycolide)/calciumphosphate (PLGA/CaP) foam matrices (OsteoScaf™) were obtained from BoneTec Corp. (Toronto, Canada). PLGA (75:25) polymer was prepared as previously described [14]. Briefly, PLGA (Birmingham Polymers, Birmingham, AL, USA) was dissolved in dimethylsulfoxide and thoroughly mixed with CaP particles. The resulting PLGA/CaP mixture was poured into a mold filled with fused glucose crystals (grain size ≈0.85-1.18

mm, Redpath Sugar, Toronto, Ontario, Canada) and transferred to a refrigerator (-18°C) to set for one hour. The PLGA was precipitated and sugar crystals leached out by immersion in double distilled H₂O (ddH₂O) at room temperature (20°C) for three days, during which time the ddH₂O was changed approximately four times per day.

In addition to the CaP phase embedded within the polymeric structure a thin surface layer of CaP was deposited onto the PLGA/CaP scaffolds (=coating) using a modified biomimetic method where the nucleation step from the biomimetic method was omitted. Samples were pre-wetted in 70% ethanol, and rinsed in deionized water. Following this the scaffold was immersed in concentrated (x1.5) simulated body fluid (SBF: Na⁺ 142.0 mM, K⁺ 5.0 mM, Mg²⁺ 1.5 mM, Ca²⁺ 2.5 mM, Cl⁻ 147.8 mM, HCO₃⁻ 4.2 mM, HPO₄²⁻ 1.0 mM, SO₄²⁻ 0.5 mM) and maintained at 37°C for a period of two days.

To incorporate porous calciumphosphate granules into the CaP coated PLGA/CaP scaffolds (=loading) a method based on a filtration technique was used. A suspension of porous particles in SBF (x1.5) was filtered through the scaffold structure. The ratio of porous particle/scaffolds weight was 60. After the filtration step scaffolds were aged in a 1.5xSBF solution for 24 hours in order to promote the bonding of the porous particles to the CaP coating. Finally, the loaded scaffolds were washed in distilled water several times until the washing water was clear and then dried in a vacuum desiccator.

Scaffold material properties

For this study biodegradable macroporous PLGA/CaP scaffolds were utilized. The porosity averaged 90% and the pores were interconnected. We used OsteoScaf™ scaffolds with a diameter of 10.0 mm and length of 12.0 mm (material properties: Table 1). Its geometry and pore size ranges mimic the architecture of human trabecular bone (Figure 3). Additional to the embedded CaP phase in the polymeric scaffold structure scaffolds consisted of CaP "coated" (and "loaded") on its surface.

Animal model

The study was approved (license number 35/9185.81-3) by the regional council of Tübingen (Germany) and is in strong accordance with the EU ISO 10993-6. Female Chinchilla-Bastard-Rabbits were used for this study. The animals were provided from a commercial breeder (Charles River, Germany). They were 6 ± 1 months of age and had a body weight of 4.2 ± 0.5 kg before surgery. The strain Chinchilla-Bastard was used because other rabbit strains common for *in vivo* testing like New Zealand White are known to be more stress-susceptible and therefore have a higher narcosis risk [15].

Study design

As described before in a previous publication on this study [7]. Chinchilla-Bastards were randomly attributed into three groups:

- 1) animals received PLGA/CaP scaffolds (OsteoScaf™, "coated")

shape	cylindrical
diameter	10.0 mm
length	12.0 mm
lactate/glycolic acid ratio	75:25
PLGA/CaP ration	1:2
pore size	800 – 1800 µm
porosity	81 – 91%

Table 1: Material properties of the utilized PLGA/CaP scaffolds.

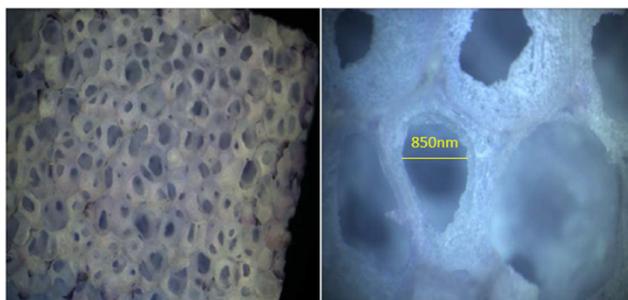


Figure 3: Scanning Electron Microscope (SEM) illustrating geometry of the utilized PLGA/CaP scaffolds, which mimic the architecture of human trabecular bone. Left picture: note the porosity of 81-91% and interconnectivity, right picture: note the pore size of 850 nm (transversal diameter, yellow line).

or “coated and loaded”) not seeded with mesenchymal stem cells,

2) animals received PLGA/CaP scaffolds (OsteoScaf™, “coated” or “coated and loaded”) seeded with MSCs, and

3) animals (control group) were sham operated, without receiving any scaffold.

X-rays were taken frequently *in vivo* to verify the maintenance of the correct location of the plate and to monitor the process of bone healing within the CSD. Animals were sacrificed after 4 and 26 weeks. Post mortem samples of the left femur containing the region of the CSD and the implanted scaffold were examined using micro-computer tomography (μ -CT) and histology (results published by [7]).

For this study a total of six samples were investigated by Raman Spectroscopy:

- scaffold “coated” before implantation
- scaffold “coated and loaded” before implantation
- scaffold “coated” 4 weeks after implantation
- scaffold “coated” 26 weeks after implantation
- diaphysis of an origin rabbit femur (without receiving surgery)
- origin embedding substance methylmetacrylate (MMA, without scaffold)

Two of the samples (c, d) that were analyzed by Raman Spectroscopy were taken from animals that received scaffolds seeded with MSCs (*group two*), and samples were previously investigated with μ -CT and histology.

Housing and animal care

The animals were housed in individual cages (floor space: 1.5 m²) with supply of water and food ad libitum at least one week before surgery to guarantee homeostasis. The animals were kept at a temperature of 21°C and a humidity of 50% according to the European Guidelines for care and use of laboratory animals. Artificial lighting was used to maintain a rhythm of 12 hours a day and 12 hours a night.

Anaesthesia

Bone marrow harvesting and scaffold implantation were performed under general anaesthesia. A dosage of 0.2 mg/kg body weight atropine (Atropin, 0.5 mg/ml, Eifelfango) was given subcutaneously before

anaesthesia was induced by injecting 10-20 mg/kg body weight ketamine (Ketamin™ 10%, Sanofi) and 1-3 mg/kg body weight xylazine (Rompun™ TS 20 mg/ml, Bayer Vital) intramuscularly. For the maintenance of anaesthesia after confirming the status of surgical tolerance 0.5-2.0 mg/kg body weight pentobarbital-natrium (Narcoren®, Merial) was supplied intravenously (i.v.). Additionally Ringer lactate solution (B. Braun) was continuously infused i.v.

Bone marrow harvesting and culture

Bone marrow was harvested by aspiration from the iliac crest of rabbits not undergoing the osteotomy and scaffold implantation procedure. The iliac crests were prepared with antiseptic solution (10% povidone iodine). An 18-gauge spinal needle was introduced into the dorsal spine and 3.0ml isotonic NaCl-solution were injected. Subsequently 2-6 ml bone marrow/rabbit were aspirated.

To obtain a precursor cell enriched fraction from the aspirated bone marrow, a Histopaque/Ficoll (1:2, v : v, density 1.077) density gradient centrifugation method was performed. The precursor cells were washed twice in phosphate buffered saline and afterwards transferred into a common cell culture flask for cultivation. Cells were cultivated at 95% humidity, 5.0 \pm 0.5% CO₂, and 37.0 \pm 0.5°C in ZKT-1 medium (Biochrom). The ZKT-1 medium was supplemented with 10% penicillin/streptomycin, 10% FCS (Invitrogen), 0.2 mM L-ascorbate, 10 mM β -glycerophosphate and 10⁻⁸ M dexamethasone (Sigma, Saint Louis, Mo) to osteogenically induce mesenchymal stem cells. Just before cells reached confluence they were seeded on scaffolds (10⁷ cells/cm³). For the cultivation of the cell-seeded scaffolds a continuously perfused miniaturized fixed-bed bioreactor system (culture medium volume: 30 ml) as described before was used. In the bioreactor the following cultivation parameters were used: 37°C, 20% PO₂, 200 μ l/min perfusion rate, 120 rotations per minute of the medium stirrer.

Scaffold implantation

For scaffold implantation the left hind limb was shaved and laved with 10% povidone iodine. The animal was positioned on the right lateral site and covered with sterile cloths. A lateral longitudinal incision of the skin was made followed by exposure of the femoral diaphysis. The periosteum was removed from the area of osteotomy using a periosteal elevator (Figure 4A). A 2.7 mm titanium seven-hole linear reconstruction plate was then adjusted to the anterolateral cortex and fixed with six 2.7 mm diameter bicortical screws, three on each side of the proposed osteotomy. An oscillating saw (Command 2 Sag Saw, Stryker Leibinger) was used for the resection of a complete piece of diaphyseal bone 12 mm long (radical critical-sized defect) under saline irrigation to prevent a temperature increase (Figure 4B). Finally, the scaffold was implanted in the defect region (Figure 4C). The wound was closed by suturing muscles, fasciae and skin separately layer by layer.

Post mortem preparation

For *post mortem* preparation the animals were anaesthetized as described before and sacrificed. The complete left femur was explanted together with adherent connective tissue and immersion fixated in 4% formalin. The ratio of tissue (mg) to fixative (ml) was 1:100 and the fixative was exchanged daily. For examination the femoral diaphysis part containing the osteotomy site was processed. Afterwards the samples were kept in water for 12 hours to wash out the formalin. Samples were then dehydrated using graded ethanol concentrations. Finally, the samples were immersed in xylol for 48 hours to remove fatty tissues.

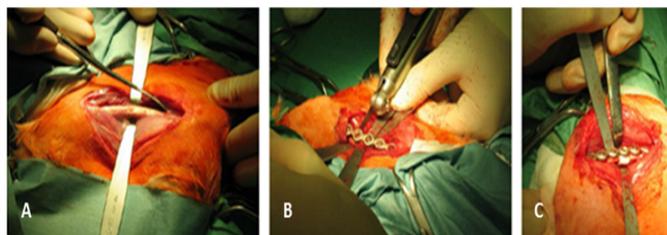


Figure 4: Implantation of the PLGA/CaP scaffold in the left rabbit femur, A: removing of the periosteum with a periosteal elevator, B: resection of a 12 mm long piece of the bone using an oscillating saw (critical-sized defect), C: implanted scaffold.

Histological examination

The samples were embedded in methylmetacrylate (MMA, Technovit® 9100, Kulzer, Bad Homburg, Germany) at 4°C using a vacuum exsiccator to remove air bubbles. They were kept at -20°C for several days until polymerization was completed. After cutting and grinding according to Hillmann et al. [16] the polymer embedded samples were stained with Toluidine Blue. For the histological analysis a “Leica DMRX-microscope” was used.

Micro-computer tomography (μ-CT)

The μ-CT analysis was performed using the “Skyscan 1072” (Skyscan, Aartselaar, Belgium). The detector-size was 1024×1024 (12 bit CCD-detector) and focus size of the X-ray tube was 8 μm. The reconstruction software was based on the cone-beam-algorithm. Before μ-CT, the metallic reconstruction plate and screws were removed carefully.

Preparation of the samples for raman spectroscopy

Samples were leached of methylmetacrylate (MMA) before examination with Raman Spectroscopy. Therefore a method previously described and recommended by the company “HeraeusKulzer” for leaching of MMA-embedded samples was utilized (Table 2).

Raman spectroscopy of samples

We used a “WITec confocal Raman microscope” (WITecfocus innovations GmbH, Ulm, Germany) of “alpha 300series” for Raman analysis of the six samples.

The Raman Microscope can be described as a device working with confocal transmission and is characterized by high sensitivity, high resolution, and wavelength optimized UHTS300 spectrometer system and single point spectrum acquisition. Therefore, molecular analysis about defined regions of any sample can be realized. Up to now it has been used particularly for geological examinations [17-19].

All Raman Spectroscopy images were studied with a laser wavelength of 633 nm. The central region surface area of the two samples from animal group two (scaffolds “coated” and seeded with MSCs) were examined, one sample 4 and one sample 26 weeks after implantation.

Results

Histology and micro-computer tomographie (μ-CT)

Shrinkage of the scaffolds did not occur 4 weeks after implantation and there were no samples showing a necrosis area in the central

chemical agent	residence time (minutes)	temperature
xylol	20 – 20 – 20	room temperature (RT)
2-methoxyethylacetat	20 – 20	RT
acetone	5 – 5	RT
deionized water	2 – 2	RT

Table 2: Leaching of methylmetacrylate (MMA) from Technovit®-embedded samples before Raman Spectroscopy.

scaffold volume (Figure 5). Functional blood vessels and angiogenesis were observed in central scaffold regions (Figure 6), affirming the consisting interconnectivity of the scaffold structure.

Animals of group two that received a scaffold seeded with MSCs showed a significantly higher degree of mineralization versus animals of group one that received a scaffold without MSCs ($p=0.041$) [7]. In samples of sham operated animals (group three) there was only a sparse of mineralization. None of the sham operated animals was able to bridge the CSD with mineralized bone tissue after 26 weeks. Further details of histological and μ-CT-results have been published before [7].

Raman spectroscopy

Raman spectrum of origin rabbit femur (without surgery): The spectrum of the origin rabbit femur diaphysis consists of peaks that are characteristic for bone tissue (Figure 7A). The peak at 960 cm^{-1} suggests the presence PO_4^{3-} as an integral part of calciumphosphate (CaP) in mineralized bone tissue. Peaks from 1200 – 1700 cm^{-1} indicate amide groups and another peak at 2900 cm^{-1} represents CH_2 links in collagen type I, both components of organic bone tissue.

Raman spectrums of scaffolds before implantation: The signals of the scaffold “coated” and the scaffold “coated and loaded” are both dominated by the peak at 960 cm^{-1} , showing the presence of PO_4^{3-} in the scaffold as it is a component within the embedded, coated and loaded calciumphosphate (CaP). Peaks which would indicate organic structures (amide groups, CH_2 links of collagen type I) cannot be found. There are no significant differences between the spectrums of both types of scaffolds (Figure 8A and 8B).

Raman spectrum of a scaffold 4 weeks after implantation: The spectrum of a scaffold “coated” and seeded with MSCs 4 weeks after implantation showed significant changes compared to the spectrum of the scaffold “coated” before implantation (Figure 9A). Most remarkable, the spike at 960 cm^{-1} does not occur here, which demonstrates the absence of any CaP in the examined region at that point of time. Specific peaks of organic bone tissue (amide groups in the range from 1200–1700 cm^{-1} , CH_2 links at 2900 cm^{-1}) reemerge.

Raman spectrum of a scaffold 26 weeks after implantation: The spike indicating mineral bone tissue (CaP) at 960 cm^{-1} can be detected again on the spectrum of a scaffold “coated” and seeded with MSCs 26 weeks after implantation. Peaks indicating organic bone tissue (amide groups in the range from 1200–1700 cm^{-1} , CH_2 links at 2900 cm^{-1}) still emerge (Figure 9B).

Raman spectrum of origin embedding substance (methylmetacrylate): The spectrum of the embedding substance methylmetacrylate (MMA) did not show any specific spikes which can be found in any of the other Raman spectrums (Figure 7B). Thus spikes originated by material artifacts from the embedding substance can be excluded.

Discussion

Histological examinations and μ-CT results confirm an excellent

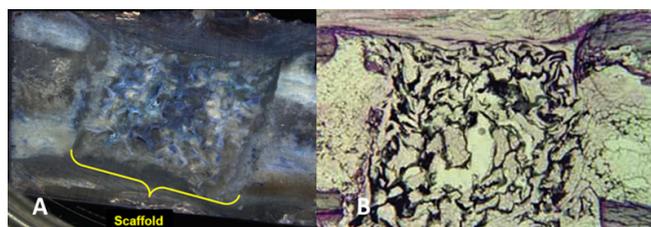


Figure 5: Scaffold coated and loaded 4 weeks after implantation in the rabbit femur (toluidine blue staining, primary magnification 1:2). A: no obvious shrinkage B: consisting structure and interconnectivity.

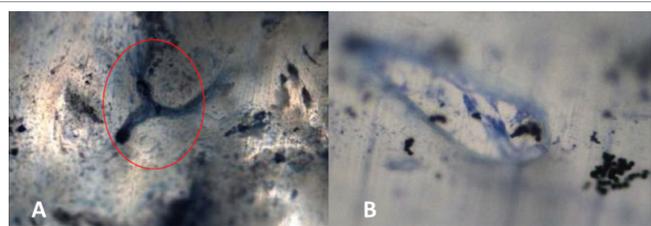


Figure 6: Central volume of a scaffold coated and loaded 4 weeks after implantation in the rabbit femur, toluidine-blue staining. A: blood vessel formation (primary magnification 1:100, epi-illumination mode). B: intussusceptive angiogenesis (primary magnification 1:500).

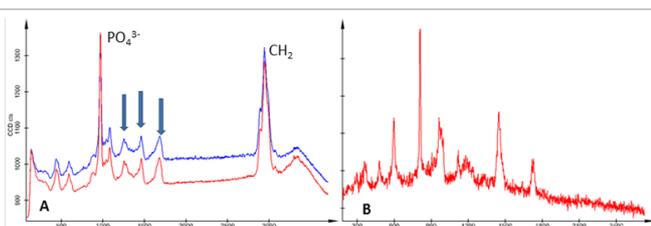


Figure 7: A: Raman spectrum of origin rabbit femur diaphysis with peaks at 960 cm^{-1} (as an indicator for mineral bone tissue), between $1200\text{-}1700\text{ cm}^{-1}$ (marked by blue arrows, amide groups of organic bone tissue) and at 2900 cm^{-1} (CH_2 links of collagen type I). B: Raman spectrum of the embedding substance methylmetacrylate (MMA).

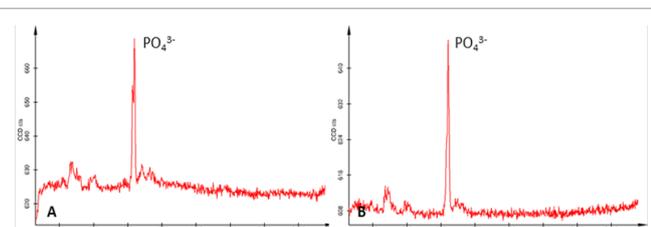


Figure 8: A: Raman spectrum of scaffold "coated" before implantation, B: Raman spectrum of scaffold "coated and loaded" before implantation. The main peak in both spectra is located at 960 cm^{-1} representing CaP.

biocompatibility and mechanical stability of the PLGA/CaP scaffolds used for Bone Tissue Engineering (BTE) in an animal model [7]. This was verified by the presence of blood vessels and mineralized bone formation in the center volume of the scaffolds (Figure 6). Fast shrinkage of scaffolds did not occur and interconnectivity of the scaffold structure persisted for many weeks (Figure 5). Central necrosis was not observed. Animals that received a scaffold had significant superior healing results and more mineralized bone tissue in the CSD region after 4 and 26 weeks, which was demonstrated by the μ -CT results as published before [7].

In this study focus was put on the question whether Raman Spectroscopy could provide additional information about the qualitative *in vivo* degradation process of PLGA/CaP scaffolds seeded with MSCs after implantation in the rabbit femur. A total of six samples were investigated by Raman Spectroscopy.

Most important for interpretation of the degradation process of the PLGA/CaP scaffolds are the observed changes in spikes within the different Raman spectrums, which provide relevant information:

In Raman spectrums of scaffolds before implantation (Figure 8a and 8b) the biggest spike could be found at 960 cm^{-1} , representing CaP embedded in and coated (and loaded) on the scaffold. As expected no signals of CH_2 and amides were seen as they are elements of organic bone tissue.

In Raman Spectrum of a scaffold "coated" seeded with MSCs 4 weeks after implantation (Figure 9a) an absence of the spike at 960 cm^{-1} was noticed, suggesting all CaP embedded in and coated on the scaffold were already degraded and dissolved at that point of time. Histological examinations 4 weeks after implantation could not demonstrate significant shrinkage of the scaffolds (Figure 5). Hence, it must be concluded that the consistency of the structural shape and interconnectivity of the scaffolds is provided by the polymer of PLGA alone without any effects of CaP on the mechanical scaffold stability, which was already dissolved at that time. Furthermore, peaks representing organic tissue noticed on this Raman spectrum (amide groups at $1200\text{-}1700\text{ cm}^{-1}$ and CH_2 links at 2900 cm^{-1} , Figure 9A), indicated the presence of organic tissue in that area.

The peak at 960 cm^{-1} as an indicator for mineral bone tissue can be seen on the Raman spectrum of a scaffold "coated" seeded with MSCs 26 weeks after implantation (Figure 9b), interpreted as *de novo* mineral bone formation at that point of time. Allocation of peaks in this Raman spectrum show great similarity to peaks in the Raman spectrum of the origin rabbit femur bone (Figure 7a). The presence of vital *de novo* bone formation (mineral and organic bone tissue) has to be assumed in the central scaffold volume 26 weeks after implantation. This assumption matches with the results of μ -CT scans of the central scaffold volume, which could illustrate the presence of bone formation in that region [7].

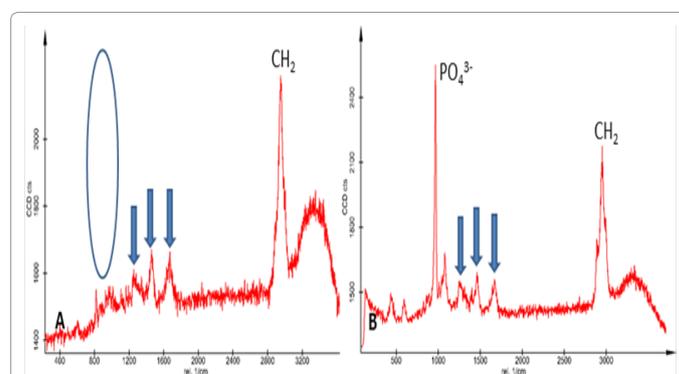


Figure 9: Raman spectrums of a scaffolds "coated", blue arrows: amide groups. A: 4 weeks after implantation. There is no spike at 960 cm^{-1} (ellipse), indicating the absence of CaP at that point of time. B: 26 weeks after implantation. Note the recurrence of the spike at 960 cm^{-1} .

Conclusion

Application of Raman Spectroscopy for investigations of PLGA/CaP scaffolds seeded with MSCs after implantation in the rabbit femur provided new information about the procedure and progress of *in vivo* degradation for this type of bone replacement material: CaP embedded in and coated on the scaffold was dissolved completely after 4 weeks, and maintenance of the scaffold's structure and interconnectivity was provided by PLGA alone. Further and modified applications of Raman Spectroscopy are supposable. Raman Spectroscopy must be considered as a valuable tool for characterization of BRM and bone tissue in the field of BTE.

Acknowledgements

We thank the European Union for financing this project. It was part of the Framework Program 5 (EU-Project G5RD-CT-200-00282 "Tissue Reactor").

References

1. Pan Z, Ding J (2012) Poly(lactide-co-glycolide) porous scaffolds for tissue engineering and regenerative medicine. *Interface Focus* 2: 366-377.
2. Salgado AJ, Coutinho OP, Reis RL (2004) Bone tissue engineering: state of the art and future trends. *Macromol Biosci* 4: 743-765.
3. Amini AR, Laurencin CT, Nukavarapu SP (2012) Bone tissue engineering: recent advances and challenges. *Crit Rev Biomed Eng* 40: 363-408.
4. Habibovic P, van der Valk CM, van Blitterswijk CA, De Groot K, Meijer G (2004) Influence of octacalcium phosphate coating on osteoinductive properties of biomaterials. *J Mater Sci Mater Med* 15: 373-380.
5. Zhu L, Liu W, Cui L, Cao Y (2006) Tissue-engineered bone repair of goat-femur defects with osteogenically induced bone marrow stromal cells. *Tissue Eng* 12: 423-433.
6. McBane JE, Sharifpoor S, Cai K, Labow RS, Santerre JP (2011) Biodegradation and *in vivo* biocompatibility of a degradable, polar/hydrophobic/ionic polyurethane for tissue engineering applications. *Biomaterials* 32: 6034-6044.
7. Endres S, Hiebl B, Hägele J, Beltzer C, Fuhrmann R (2011) Angiogenesis and healing with non-shrinking, fast degradable PLGA/CaP scaffolds in critical-sized defects in the rabbit femur with or without osteogenically induced mesenchymal stem cells. *Clinical Hemorheology and Microcirculation* 48: 29-40.
8. Wang W, Li B, Li Y, Jiang Y, Ouyang H, et al. (2010) *In vivo* restoration of full-thickness cartilage defects by poly(lactide-co-glycolide) sponges filled with fibrin gel, bone marrow mesenchymal stem cells and DNA complexes. *Biomaterials* 31: 5953-5965.
9. Yang Y, Zhao Y, Tang G, Li H, Yuan X, et al. (2008) *In vitro* degradation of porous poly(l-lactide-co-glycolide)/ β -tricalcium phosphate (PLGA/ β -TCP) scaffolds under dynamic and static conditions. *Polym Degrad Stabil* 93: 1838-1845.
10. Bertoluzza A, Fagnano C, Monti P, Simoni R, Tinti A, et al. (1992) Raman spectroscopy in the study of biocompatibility. *Clin Mater* 9: 49-68.
11. Taddei P, Tinti A, Bottura G, Bertoluzza A (2000) Vibrational spectroscopic characterization of new calcium phosphate bioactive coatings. *Biopolymers* 57: 140-148.
12. De Grauw CJ, Otto C, Greve J (1997) Line-Scan Raman Microspectrometry for Biological Applications. *Appl Spectrosc* 51: 1607-1612.
13. Penel G, Delfosse C, Descamps M, Leroy G (2005) Composition of bone and apatitic biomaterials as revealed by intravital Raman microspectroscopy. *Bone* 36: 893-901.
14. Guan L, Davies JE (2004) Preparation and characterization of a highly macroporous biodegradable composite tissue engineering scaffold. *J Biomed Mater Res A* 71: 480-487.
15. Haberstroh J, Henke J (2003) Anästhesie beim Kaninchen in: Anästhesie und Analgesie beim Klein- und Heimtier. Schattauer Verlag, Stuttgart.
16. Hillmann G, Hillman B, Donath K (1991) Enzyme, lectin and immunohistochemistry of plastic embedded undecalcified bone and other hard tissues for light microscopic investigations. *Biotech Histochem* 66: 185-193.
17. Rotundi A, Baratta GA, Borg J, Brucato JR, Busemann H, et al. (2008) Combined micro-Raman, micro-infrared, and field emission scanning electron microscope analyses of comet 81P/Wild 2 particles collected by Stardust. *Meteorit Planet Sci* 43: 367-397.
18. Steele A, Fries MD, Amundsen HEF, Mysen BO, Fogel ML, et al. (2007) Comprehensive imaging and Raman spectroscopy of carbonate globules from Martian meteorite ALH 84001 and a terrestrial analogue from Svalbard. *Meteorit Planet Sci* 42: 1549-1566.
19. Toporski J, Fries M, Steele A, Nittler L, Kress M (2004) Sweeping the skies: Stardust and the origin of our Solar System. *Imaging & Microscopy* 38-40.
20. Smith R, Rehman I (1994) Fourier transform Raman spectroscopic studies of human bone. *J Mater Sci-Mater M* 5: 775-778.