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ε-Caprolactone in Micro-Chambered Ceramic Beads – A New Carrier for Gentamicin

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Key Words
ε-Caprolactone · Micro-chambered ceramic beads · Gentamicin

Abstract
Purpose: The purpose of this preliminary and descriptive study was to evaluate a biodegradable drug delivery system in combination with an innovative ceramic implant. ***Methods:*** The delivery of gentamicin of standardized samples was measured in the laboratory using ultra-high-performance liquid chromatography. Biocompatibility and biodegradation of the materials was investigated in an animal experiment in sheep up to 14 months. As carrier ε-caprolactone, 1:1 mixed with gentamicin, intruded into micro-chambered β-tricalcium-phosphate beads (MCB®) was studied. ***Results and Discussion:*** Gentamicin was released in calculable concentrations during the first 30 days. The release from ε-caprolactone was higher than that from polymethylmethacrylate and more predictable. The caprolactone carrier was reabsorbed by osteoclasts.

Introduction

Background

Ceramic bone void fillers for bone augmentation and other implants present two problems: bacterial contamination and the lack of bone marrow and vascularized tissue for an active antibacterial defense. To protect orthopaedic implants, measures to prevent bacterial contamination during the intervention were undertaken, including double gloves and solutions containing antibiotics for the lavage of the wound during the operation and before closing [1]. Nevertheless, up to 3% of the implants are contaminated with bacteria, yielding acute or chronic osteomyelitis, the path of which was considered a haematogenous infection [2–5]. Consequently, experiments were started to protect the implants with antibiotic-loaded polymers [2, 6–8], and drug delivery for the local protection of implants was born.

Objectives

Antibiotic-loaded polymethylmethacrylate (PMMA) bone cements used for drug delivery in orthopaedic surgery present a non-resorbable polymer material, and its release is related to the overall surface area [9]. Based on these findings, the question arose whether a biodegradable material could replace the PMMA polymer releasing the antibiotic over a reasonable time span. Attempts to coat ceramic implants were performed with different polymers and waxes applying a plunging procedure [10–12]. Coating the otherwise active calcium phosphate surface yielded a loss of osseointegration of the implants [13].

Since the release of antibiotics follows a diffusion process of water-soluble substances from polymers which in-

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corporate water in molecular form [14], all biodegradable polymers, i.e. polyesters, are principally suited to act as a drug delivery system for antibiotics.

Micro-chambered ceramic beads (MCB) primarily form a lamellar cancellous bone scaffold. Due to their capillary forces, they provide the release of bone morphogenetic protein (BMP) bound to collagen type I [15]. ε-Caprolactone has been proven to be biocompatible and biodegradable as a substance [16] and in combination with β-tricalcium phosphate (β-TCP) [17]. The polymer has been used for many years in clinical and dental practice as a barrier membrane to promote bone regeneration [18] and as root-filling material [19–21].

Purpose of the Study

The purpose of this study was to show that intrusion of a defined mass of a biodegradable carrier for gentamicin into pores of the micro-chambered β-TCP ceramic beads preserves osteoconduction and colonization by osteoblasts of the calcium phosphate surfaces on the one hand and, on the other hand, provides local protection against bacterial contamination, effectively releasing gentamicin sulphate over a certain period of time. The biocompatibility and biodegradability of ε-caprolactone should be followed histologically.

Material and Methods

The study was performed in 2 steps: laboratory evaluation of gentamicin release kinetics in vitro, followed by an animal experiment in sheep.

Laboratory Experiment

Gentamicin (Fujian Fukang, Fuzhou, China) was used as a sulphate, an antibiotic on the basis of aminoglycosides. ε-Caprolactone (BASF, Ludwigshafen, Germany) comprising a molecular weight of 50,000 was chosen as carrier. The composite material was processed with the help of a ball mill resulting in a 1:1 mixture of gentamicin sulphate and ε-caprolactone. The mixture was kneaded in an industrial compounder and extruded into a cylindrical tool, resulting in cylinders measuring 5 mm in diameter which are pelletized by a machine to a length of 5–6 mm. The weight of the samples ranged from 96.33 to 148.06 mg, with a mean value of 115.18 mg (n = 20). Six samples were taken for the experiment and stored in phosphate-buffered solution (pH 7.0). Five samples were measured daily with ultra-high-performance liquid chromatography (UPLC; Waters, Milford, Mass., USA) over a period of 3 days, and 4 samples were measured again after 10 days. Two samples were measured daily over a time span of 10 days, whereas 4 samples were continuously held in the experiment and were measured after 10, 20 and 30 days, respectively. The assay was performed with UPLC (Waters) equipped with a column of 2.1 × 100 mm (ACQUITY UPLC® HSS C18, 1.8 μm). The eluent was acetonitrile/water 70:30 (v/v) and the detector used was Waters FLR Fluoroscence detector, equipped with an Hg-Xe lamp (excitation 260 nm, emission 315 nm). For calibration, 5 standard solutions were prepared: 0.1, 0.25, 0.5, 1.0 and 2.0 μg/ml. For the control of the 5 standard solutions, a control solution of 0.5 μg/ml from stock solution 'A' was prepared. Stock solution 'A' comprised a concentration of 0.4 μg/ml and was prepared with 20 mg gentamicin sulphate, dissolved in 50 ml buffer solution at pH 7.0 (Merck Certipur®; Merck KGaA, Darmstadt, Germany).

To make gentamicin detectable for the fluorescence detector, the standard samples and the samples to be analyzed were derivatized with 3.0 equivalents of 9-fluorenylmethyl chloroformate (Merck) in Certipur buffer solution (Merck). The calibration curve was designed accordingly. The ε-caprolactone/gentamicin sulphate pellets were rinsed in 50 ml buffer solution at pH 7.0 (Certipur). A solution of 2.0 μl was derivatized with 3.0 equivalents of 9-fluorenylmethyl chloroformate (Merck) in buffer solution (Certipur) at pH 8.90 and measured with UPLC. Quantification was carried out with Empower® software (Waters). Data of the release were graphically displayed and superposed.

Gentamicin molecules are built with the following 3 hexosamines: gerosamine, 2-deoxy-streptamine and purpurosamine, forming the gentamicin molecules of the 'type C' group [22, 23].

Animal Experiment

Ten female sheep were operated on with a standardized and very precise cylindrical defect measuring 9.4 × 20 mm in diameter and depth, respectively. The patellar groove model using the wet-grinding diamond technology was applied: the defect was placed in the centre of the patellar groove using a drill guide [24]. A combination of 12.5 mg ε-caprolactone with 12.5 mg gentamicin sulphate was intruded into the 6-mm beads. The defects were filled with 6- and 4-mm micro-chambered β-TCP beads (fig. 1a–e). Outward diffusion was prevented using a 1-mm-thick press-fit-inserted solid ceramic β-TCP lid (fig. 1f). Investigations were performed at 6 weeks (3 animals) and 2 and 3 months (3 animals). One animal was kept in reserve and sacrificed after 14 months. Histology of non-demineralized sections was evaluated after perfusion fixation via the femoral artery with Karnovsky solution and drainage via the femoral vein, followed by a casting of the whole vasculature using an acrylate resin. The distal femurs were dissected and trimmed for CT scan as well as scanned parallel to the patellar groove at slices of 15 μm (μCT 40; Scanco Medical, Brüttisellen, Switzerland). Subsequent to the μCT, the cylinder was dehydrated, stained with basic fuchsine and embedded in MMA. The hardened bloc was cut into serial sections of 500 μm parallel to the patellar sliding groove (fig. 2). The cross-sections were ground with corundum paper to 110 μm, micro-radiographed in the Kristalloflex 710 (Siemens), further thinned to 50 μm and covered on glasses. Documentation was performed with a Leitz Orthoplan microscope using transmitted light on Kodak Ektachrome Professional 'T' films for light sources with 3,500 K.

Results

Laboratory Results

The pharmaceutically applied component of gentamicin sulphate measured in our series contained gentamicin type C (C_1, C_{1a}, C_2, and C_{2a}). The 4 components showed a characteristic chromatogram in our calibration series (fig. 3a).

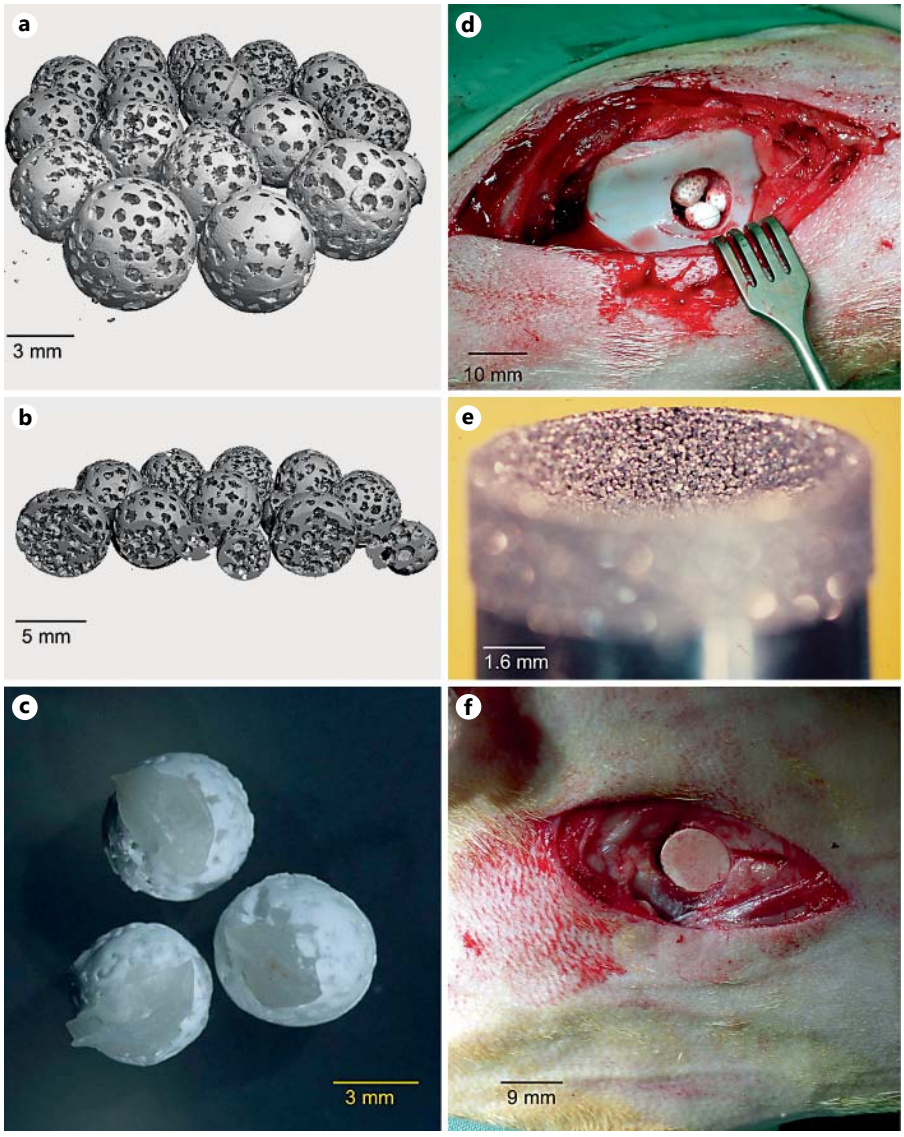

Fig. 1. a Fully interconnected MCB of β-TCP measuring 6 mm in diameter and providing 85% porosity and 99% purity. Three-dimensional reconstruction of μCT slices (μCT 40; Scanco Medical). **b** Frontal section through the beads in 03 **a** (μCT 40; Scanco Medical). **c** β-TCP beads revealing intruded ε-caprolactone; most of the ceramic surface remains accessible for covalent links of type I collagen fibres. **d** Operating site of the patellar groove of the sheep presenting the defect filled with MCBs. **e** Diamond instrument: the cylindrical cutting tool is coated with diamonds. **f** Operating site: the defect containing the MCBs combined with ε-caprolactone and gentamicin covered by a ceramic lid.

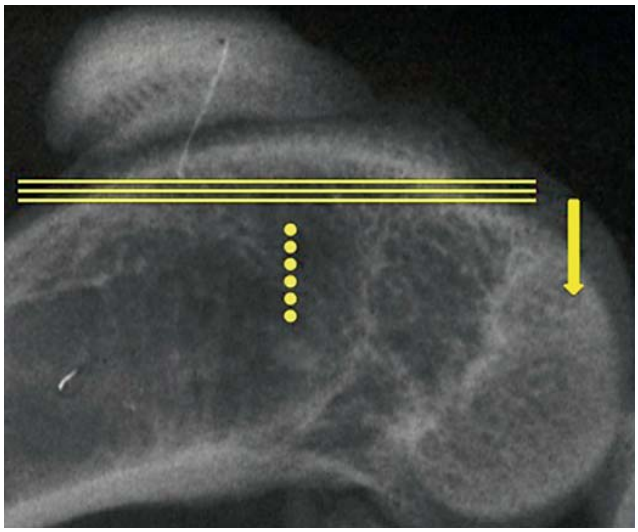

Fig. 2. Lateral view of an X-ray of the sheep knee joint indicating the cross-sections processed for histology. Arrow indicates the direction of the series of cuts.

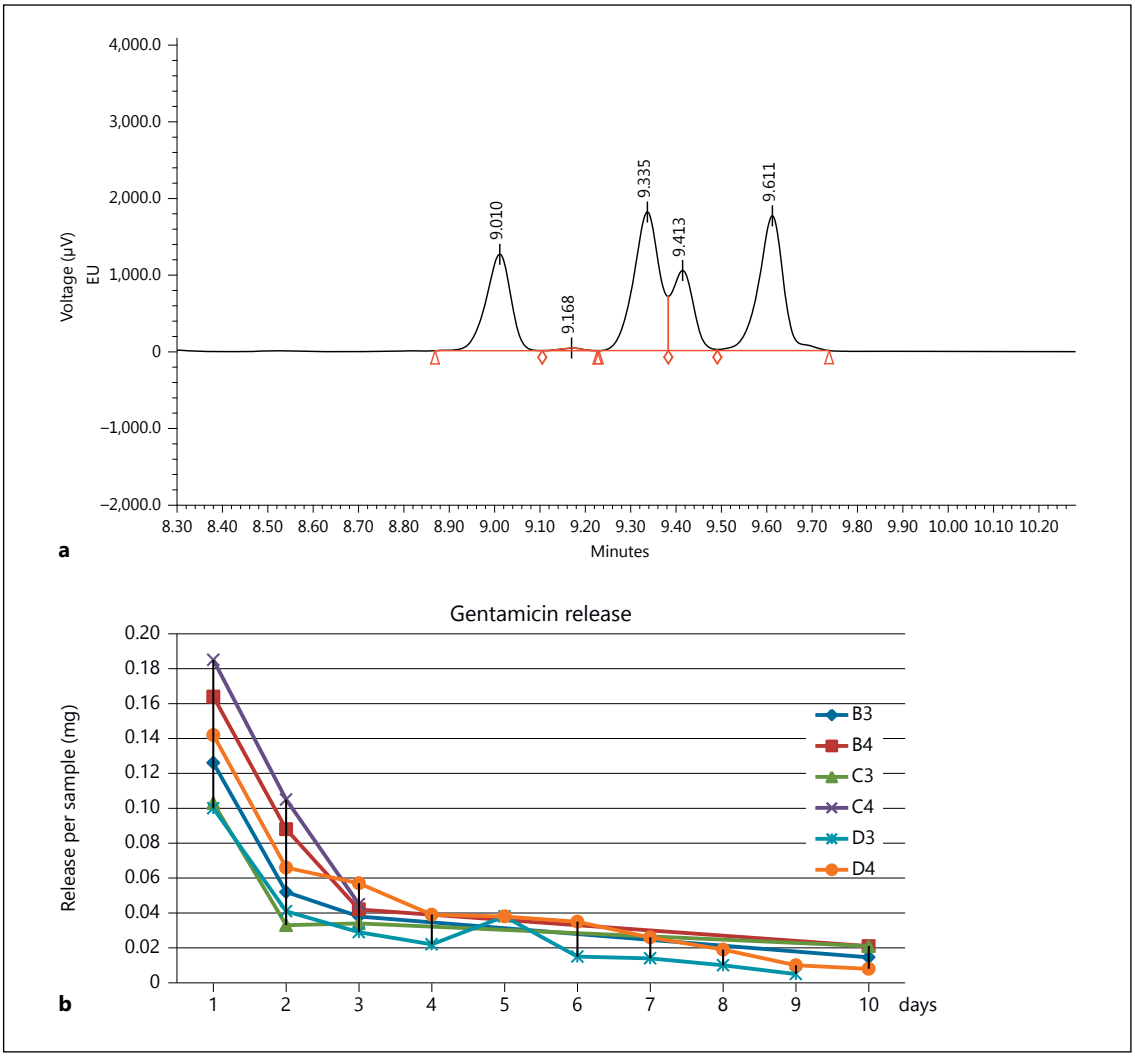

Fig. 3. a Chromatogram of gentamicin with the 4 characteristic peaks of the gentamicin sulphate components, taken from this study (UPLC; Waters): C_1: 9.01; C_{1a}: 9.33; C_2: 9.41 and C_{2a}: 9. **b** Release of gentamicin in 6 samples during the first 10 days, measured with UPLC (Waters).

The release of the samples showed the typical curve of gentamicin progression after 24, 48 and 72 h and after 10 days; the complete daily release is presented in figure 3b and in table 1. Two samples remained in the experiment and were measured after 20 and 30 days. The daily release in both samples was 0.5 mg/day, and the total amount over 30 days was 34.7 and 38.3 mg, respectively (table 1). The curves showed graphically identical characteristics.

Results from the Animal Experiment

No infection occurred. The intrusion of ε-caprolactone resulted in an equal filling of the centre of the ceramic beads, leaving the calcium phosphate surface accessible for colonization by osteoblasts and yielding a bactericide delivery of gentamicin during the first 10–30 days, calculated based on in vitro release.

Six weeks after implantation, β-TCP had been substantially reabsorbed, whereas ε-caprolactone was nearly unattached and clearly marked like a grape inside the pores of the MCB. The radiolucent polymer could be visualized in all µCT slices (fig. 4a). Three months after implantation, only residues of the polymer material were detected in the cross-sections, stained with alkaline fuchsine (fig. 4b–d). ε-Caprolactone particles revealed Howship-like lacunae over its entire surface and multinuclear giant cells attached to it. The material was embedded

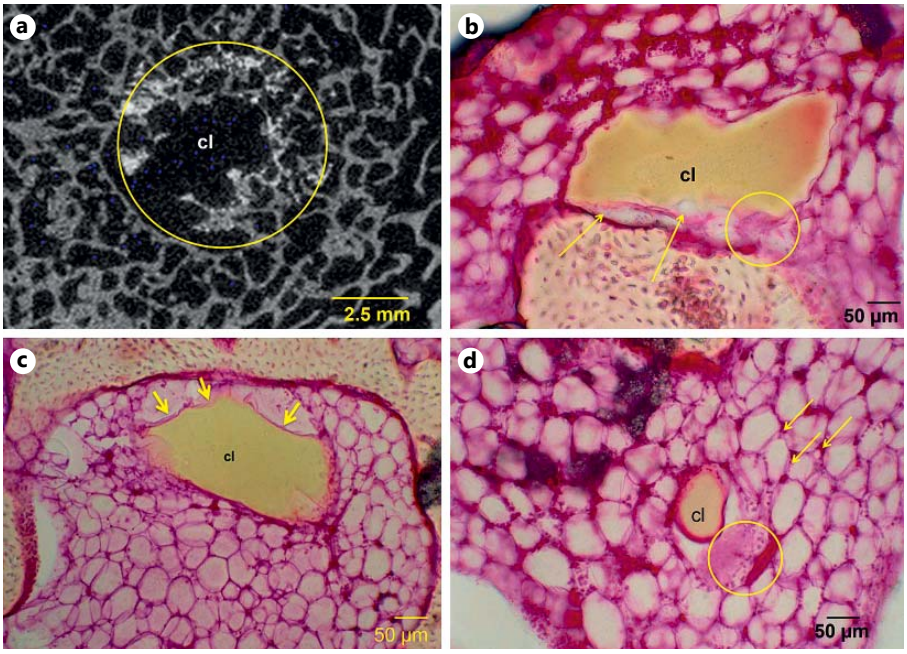

Fig. 4. **a** Nearly re-absorbed MCB (circle) with intruded ε-caprolactone (cl), mixed 1:1 with gentamicin sulphate. µCT slice through the ε-caprolactone carrier and the centre of the MCB (circle) (µCT40; Scanco Medical). **b** Residue of ε-caprolactone (cl) revealing Howship-like lacunae (arrows) and multinuclear giant cells. Biocompatible reaction with the fat marrow environment. 10.0 Leitz Orthoplan Apochromat; oil immersion; alkaline fuchsine staining; 3-month stage. **c** Thin cross-section showing minor residues of ε-caprolactone, 3 months postoperatively. The lacunae (arrows) present the remains of macro-phagocytic degradation of ε-caprolactone (cl). 10.0 Leitz Orthoplan Apochromat oil immersion; alkaline-fuchsine staining. **d** Three months after the operation, only small amounts of ε-caprolactone (cl) are found; the thin slice reveals a multinuclear giant cell (circle). The β-TCP ceramic is reabsorbed and partly phagocytized by reticulum cells (arrow). 10.0 Leitz Orthoplan Apochromat oil immersion; alkaline fuchsine staining.

Table 1. Gentamicin release over 30 days

Sample	Gentamicin, mg	Release after 10 days, mg	Release after 10 days, %	Release after 30 days, mg	Release after 30 days, %
B3	58.21	23.95	41.14	34.80	59.78
B4	58.46	27.79	47.54	38.31	65.53
C3	51.43	14.80	28.77	23.73	46.15
C4	48.17	27.73	57.57	34.73	72.09
D3	56.42	17.54	31.09	–	–
D4	54.73	24.19	44.20	–	–

within inactive fat marrow, and direct contact with bone was in part pronounced (fig. 4b); there were no signs of a granulomatous reaction. Compared with Howship's lacunae on bone, the cavities on the ε-caprolactone surface were flat and large (fig. 4b, c). At the same time, a nearly complete reabsorption of β-TCP could be observed. After 3 months, the last minor residues of β-TCP could be seen within the marrow. The reticulum cells showed phagocytized β-TCP particles everywhere within the cells, whereas residues of ε-caprolactone were rarely found at that stage (fig. 4d).

The caprolactone carrier was reabsorbed by osteoclastic reabsorption revealing lacunae known from bone remodelling. Minor residues could be found after

3 months. However, after 1 year, ε-caprolactone was no longer detected. A restitutio ad integrum was found after that time.

Discussion

The question whether a biodegradable material could replace the PMMA polymer as a carrier for antibiotics has to be discussed comparing the results of this study with the measured release of gentamicin-loaded bone cements in the literature.

Limitation of the Study

The number of samples used was not very high. There was no direct comparison with samples of antibiotic-loaded bone cements. These are ongoing studies. Validation of the mixing and kneading procedure has not yet been fully completed, and there might still be some conglomerates of gentamicin within the sample which are thus responsible for single peaks in the release curve. Standardization of the samples itself has not yet been perfected. However, the main principles of the release and the biocompatibility of the carrier and its resorption could already be presented.

Comparison with Bone Cements Loaded with Antibiotics

In most studies involving antibiotic-loaded bone cements, the assay comprises microbiological analyses measuring the area of inhibited growth in bacterial cultures [9, 25]. At the beginning of these investigations, concentrations of gentamicin were estimated by the agar-diffusion test [14]. Those assays showed low preciseness and were time consuming. The modern assays use liquid chromatography as the standard method. However, different methods can be applied. The UPLC of our study (Waters) is based on a fluorescence detector, and therefore, the samples have to be derivatized with 9-fluorenylmethyl chloroformate (Merck). The derivatization step is not necessary with the evaporative light scattering detector system [23].

With regard to antibiotic-loaded PMMA bone cements, it is known that antibiotics diffuse out of the polymer in bactericide concentrations; this was found for oral drug delivery systems as well [26]. The diffusion of water-soluble antibiotics is based on the uptake of water in molecular form, namely up to 5% as time goes on [27, 28]. The amount of the released drug is proportional to the surface area accessible for the body fluid [25, 29], the amount of antibiotics in the cement mass and the turnover of the fluid around the cement implant [25, 30]. The delivery from antibiotic-loaded bone cements depends directly upon the homogeneity of the mixture and the free surface of the PMMA implant [31, 32]. According to the surface-related release, the morphology and the dimensions of the samples are decisive [33]. The release from carriers is extensively studied in different bone cements loaded with different antibiotics [9, 14].

In most studies, quantitative measurements differ greatly; however, they confirm that during the first 24 h, and most pronouncedly during the first and second hour, a steep curve is reported, revealing release rates of 2.3–11% [25, 34, 35].

Delivery of gentamicin is more precise and predictable in the form of ε-caprolacton than in the bone cement form, which differs greatly from sample to sample and is also influenced by the mixing procedure [36]. The delivery from ε-caprolacton shows a steep drop during the first 2 days, comparable to that of bone cement and gentamicin PMMA beads [14, 32, 37]. However, it continues steadily over the following 8 days and then during the first months, as shown in the release curve of the study. Since the polymer is decomposed in vivo, the entire amount of gentamicin is delivered during the first 3 months [15].

Quantitative analysis is now standardized, i.e. the eluate is analyzed and the amount of gentamicin is defined in μg/ml. The procedure for quantification has become more precise [23].

During the first 10 days, the release from PMMA bone cement has been measured as 2.1 μg/g. The release from ε-caprolactone in our series has subsequently been measured as 4.4 μg/g.

The explanation is given by the water uptake of ε-caprolactone, its degradability and its molecular weight, which is comprised of shorter polymer chains. The difference within the number of samples might be explained by the inhomogeneity of the mixture, which had not been fully validated at that time. There is thus a slightly higher release measured of the 'D3' sample at the fifth day. Compared with the release curves of PMMA bone cements [9, 25, 37], the values in this study are very constant. With the degradation of ε-caprolactone, all gentamicin is released after 3 months, whereas the antibiotic-loaded bone cements showed release even after 5 years [9]. Even if sensitization is denied [36], there are reports of cement samples from chronically infected cemented hip components infected by fungi [38].

Due to the growing resistance against *Staphylococcus aureus* strains, new combinations of antibiotics are requested [39, 40].

Biocompatibility and Resorption of ε-Caprolactone

The bioactivity of β-TCP ceramics is well known from different animal studies and clinical evaluations [41, 42], and the bone-forming element for a cancellous bone scaffold was considered to be the ceramic bead [43]. In combination with BMP-7, it was found that bone formation, remodelling and resorption are enhanced tremendously [15]. Biocompatibility was proven in all studies [44]. ε-Caprolactone is used for vascular tissue engineering [45], tissue culturing with chondroblasts [46], in in vitro and in vivo bone regeneration [47–49], peripheral nerve regeneration [50] and skin tissue engineering [51], as barrier membrane in periodontology [52] and, for many years, as root-filling material in dentistry [19, 20, 53, 54]. Biocompatibility has been proven in all experimental and clinical studies [21]. The combination with antibiotics is not new [10] as is its combination with hydroxyapatite [55] and β-TCP [11].

However, the combination with MCBs for primary bone formation, even in combination with BMP-7, was a completely new approach for fast and physiological bone regeneration, requested for bone augmentation for which antibiotic protection is of utmost importance [15].

In conclusion, intrusion of a biodegradable carrier into ceramics preserved the bioactivity of the ceramic and provided a predictable release of the antibiotic for at least 1 month.

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