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Glycerol-L-lactide coating polymer leads to delay in bone ingrowth in hydroxyapatite implants

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Abstract

Glycerol-L-lactide as coating polymer for the delivery of basic fibroblast growth factor (bFGF) from hydroxyapatite (HA) ceramic implants was shown to lead to significant delay in bone ingrowth into the implants compared to implants without the coating polymer. The purpose of this work was to study bone ingrowth in HA ceramic implants with and without the coating polymer but without growth factors to enable differentiation between a locking effect of the pores by the polymer and the fact of inactivation of the growth factors by the polymer, which could both be possible for the delay. A defect was created in the subchondral region of both femurs in 24 miniature-pigs and was either filled by the HA implants with or without the coating polymer. Histomorphometry showed a significant delay in bone ingrowth in the polymer coated implants both after 6 and 12 weeks. Detailed histology revealed that the HA pores were completely "locked" by the polymer leading to complete loss of the osteoconductive properties of the HA. Also electron microscopy showed filling of the HA pores by the polymer. Therefore, it can be concluded that glycerol-L-lactide should not be used to coat HA ceramic implants due to significant delay in bone ingrowth.

Keywords: Hydroxyapatite coating; Polylactid acid; Drug release; Drug delivery; Bone ingrowth

1. Introduction

Delivery of growth factors to enhance fracture and bone defect healing remains a challenge in orthopaedic and in trauma surgery. Hydroxyapatite (HA) ceramics have also gained attractiveness as carrier for

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growth factors in this field. These composite materials combine the osteoconductive properties of the HA ceramics with the osteoinductive activity of the growth factor [1]. Several studies showed that HA might provide a suitable delivery scaffold for insulin-like growth factor-I (IGF-I) [2] and TGF-β [3] to enhance bone healing. The extensive work of Ripamonti et al. [4–8] have shown that bone morphogenetic proteins (BMPs) can be released by HA ceramic carriers and the application of this BMP-HA composite results in favourable new bone formation in critical size defects. Basic fibroblast growth factor (bFGF) has also proven promotive effects on bone and cartilage formation in fracture healing [9] and bone induction in demineralized bone matrix [10,11]. Wang and Aspenberg [12] and Wippermann et al. [13] showed that bFGF-coating of HA ceramics promoted bone ingrowth into the implant. The authors of the current study recently reported about significant enhancement of bone ingrowth into bFGF-coated HA implants compared to uncoated HA cylinders in miniature-pigs both after 42 and 84 days [14]. However, focal disturbance of bone ingrowth was observed which was attributed to inhomogeneous distribution of bFGF with the ceramic implants. Therefore, a coating polymer based on glycerol-L-lactide was used to achieve more homogenous release of bFGF from the HA ceramic. Encouraging in vitro release kinetic with a more homogenous release of bFGF from implants that were coated with bFGF and glycerol-L-lactide compared to pure bFGF-coating were found [15]. However, bone ingrowth was found to be significantly delayed in HA implants coated with the glycerol-L-lactide bFGF composite compared to pure bFGF-coating of the HA cylinders. Two possible reasons were suggested for this unexpected phenomenon. First, "locking" of the HA pores due to delayed degradation of the coating polymer could have led to a loss of the osteoconductive properties of the HA ceramic. Second, the use of acetone as solvent agent for glycerol-L-lactide coating polymer could have inactivated bFGF with a subsequent loss of the bFGF activity.

The purpose of this work was to study bone ingrowth and new bone formation in HA implants with the glycerol-L-lactide coating polymer but without the use of bFGF. Therefore, a potential deactivation effect on bFGF was excluded and only osteoconductive properties of the implants were studied. HA cylinders with and without the coating polymer were used in the same miniature-pig model as for the two other studies to analyze differences in bone ingrowth and new bone formation in the implants. In case of a significant difference between HA implants with and without the coating polymer by negative side effects of the polymer on the osteoconductive properties of the HA ceramic, this phenomenon will also have been responsible for the significant delay of bone ingrowth of bFGF-polymer coated cylinders in the former study.

2. Materials and methods

2.1. Hydroxyapatite

The same HA material as for the two previous studies was used [14,15]. In brief, the porous HA ceramic (Endobon®; Biomet Merck, Darmstadt, Germany) consists of sintered (1200 °C for 5 h) hydrothermally defatted and calcinated bovine cancellous bone. Endobon® has a foam-like trabecular macrostructure with interconnecting macro- and micropores (porosity: 45%–85% by volume). Cylinders with a diameter of 9.55 mm and a length of 10 mm were used as implants.

2.2. Coating polymer

Ring-opening polymerization of L-lactide with a molar ration of glycerol and L-lactide of 1:12 and 0.5% phosphoric acid as catalyst led to synthesis of the coating polymer (patent EP 0 290 983 B1). Synthesis war performed at 195 °C for 5 h. Gel-permeation chromatography revealed a molecular weight of the polymer of M_n=2612 and M_w=3845. Glass transition temperature was t_g=31.3 °C measured by differential scanning calorimetry.

2.3. Coating technique

To achieve a thin and homogeneous coating film, the HA cylinders were dipped into a solution of the polymer in a volatile solvent. This solution was made by dissolving 8 g of the glycerol-L-lactide polymer in 26.6 ml acetone. The subsequent sterile filtration was made with a ready-to-use PTFE filter cartridge with a pore size of 0.22 μm. After drying under vacuum for 12 h the cylinders were packed and stored at 4 °C.

2.4. Study design

24 male 2-year old miniature-pigs underwent surgery with insertion of pure HA ceramic and polymer-coated HA cylinders into the subchondral knee region of one side, respectively. Twelve animals were sacrificed after 42 days and 84 days, respectively. Thus, a total of 12 uncoated and 12 coated implants could be analyzed regarding bone ingrowth after 6 and 12 weeks, respectively.

2.5. Surgical technique

The same technique as for the two other studies was used [14,15]. In brief, a cylindrical defect with a size of 9.5 mm in diameter and 10 mm in height was created in the subchondral region of each femur using a saline cooled trephine (DBCS®; Biomet Merck; Darmstadt, Germany) to prevent heat necrosis of the surrounding host bone. The cylinders were press-fitted in the defects. This press-fit implantation technique allowed full weight-bearing after surgery.

2.6. Fluorochrome labeling

To study dynamic bone formation sequential quadruple fluorochrome labeling by subcutaneous administration of oxytetracycline (day 6 to 12; dose: 20 mg/kg bodyweight), Alizarin Red (day 13 to 19; dose: 25 mg/kg bodyweight), Calcein Blue (day 20 to 26; dose: 25 mg/kg bodyweight) and Calcein Green (day 27 to 33; dose: 20 mg/kg bodyweight) was performed. The pigs that were sacrificed only after 84 days obtained an additional daily injection of tetracycline between day 76 to 82.

2.7. Histology and histomorphometry

Before harvest of the lower extremities perfusion with Karnowsky's solution (4% paraformaldehyde and 2% glutaraldehyde in PBS, pH 7.4) was done via the abdominal aorta [15] to achieve better quality of fixation.

The distal femoral region was embedded in Technovit (Technovit 7200 VLC®, Kulzer, Friedrichsdorf, Germany) without decalcification. For histology sections of 50 μm were created using the "exact method" by Donath and Breuner [16].

Sections with a thickness of 500 μm were scanned for histomorphometry. The fluorochrome labeled area for the respective observation period was marked (day 42: Oxytetracyclin, Alizanin Red, Calcein Blue, and Calcein Green; day 84: entire fluorescence stained area) and assessed semi-quantitatively for new bone formation by relating the area of new bone formation to the total size of the implant using a digital image reader software (Biocom 5000®; Biocom, Les Ulis, France).

2.8. Statistics

Statistical analysis was done by paired Student's *t*-test (SPSS® for Windows 11.5, SPSS Software GmbH, Munich, Germany). Differences were considered to be significant in case of p-value < 0.05.

2.9. Scanning electron microscopy

Pure and glycerol-L-lactide-coated HA cylinders which were not coated with carbon were investigated in a Leo Supra 35 scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

3. Results

3.1. Histomorphometry

Bone ingrowth was significantly delayed in the glycerol-L-lactide coated implants compared to pure HA cylinders both after 42 and 84 days ($p = 0.02$ and $p = 0.004$, respectively) (Fig. 1). Only minimal new bone formation from the surrounding host bone was found for the HA cylinders coated with glycerol-L-lactide polymer after 42 days (mineralized area: $4\% \pm 2.5\%$). For pure HA cylinders higher bone ingrowth rate was observed after 42 days (mineralized area: $23\% \pm 5\%$) originating homogeneously from the surrounding bone stock of the femoral epiphysis. For the 42 days observation period there was a significant delay in bone ingrowth for the glycerol-L-lactide implants compared to the uncoated ones ($p = 0.02$).

After 84 days bone ingrowth into the implant was almost complete in the uncoated pure HA implants (mineralized area: $89\% \pm 9.5\%$). In the glycerol-coat-

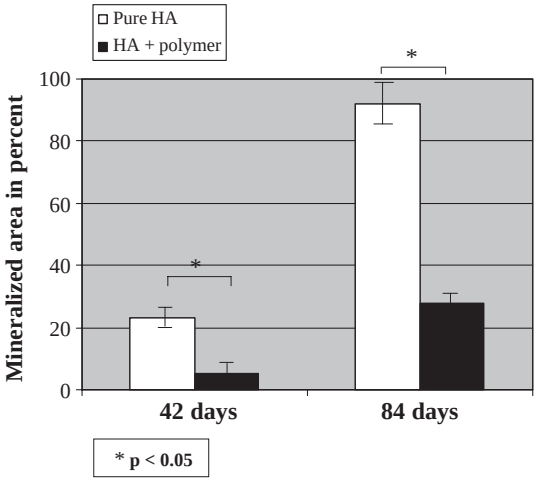

Fig. 1. Histomorphometrical analysis. The newly formed mineralized tissue was measured as percentage of the entire implant area. Both after 42 days and 84 days there was a significant difference between the pure HA cylinders and the glycerol-L-lactide-coated HA implants.

ed HA new bone formation increased after 84 days (mineralized area: 28% ± 5%) compared to the situation after 42 days. However, there was still a significant delay in relation to the uncoated implants ($p = 0.004$).

3.2. Histology

In pure HA cylinders homogenous new bone formation was found starting from the bone–implant interface (42 days, Fig. 2a) into the centre of the implant (84 days, Fig. 2c). The different fluorescence markers revealed time-dependent abundant bone apposition in the HA pores onto the ceramic itself.

The glycerol-L-lactide group showed comparable bone activity in the surrounding host bone area after 42 days (Fig. 2b). However, only minimal bone ingrowth into the implant itself was seen after 42 days. Histology showed a distinctive filling of the HA pores by the polymer appearing as "locking substance" for

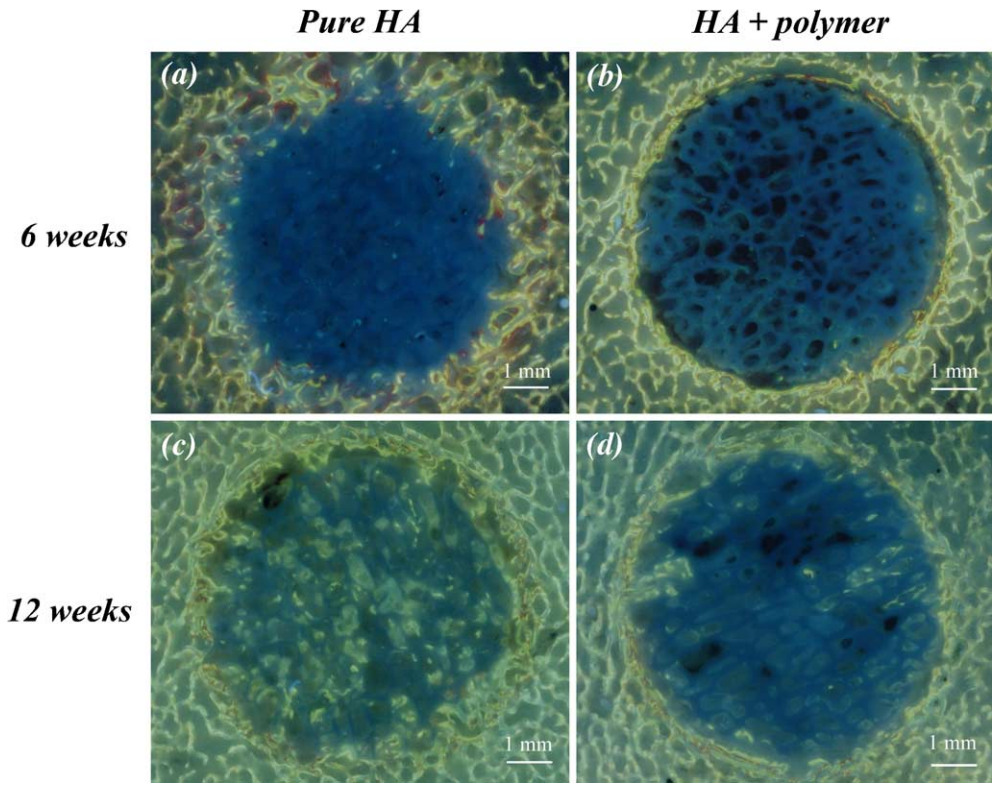

Fig. 2. Histological overview over bone ingrowth into pure HA and into glycerol-L-lactide-coated HA implants. Distinctive better bone ingrowth could be seen in pure HA cylinders compared to glycerol-L-lactide-coating both after 42 and 84 days.

bone ingrowth from the adjacent femoral bone stock (Fig. 3a). The "activated" surrounding host bone seemed to try to "invade" the implant but was unable to do this due to the "locked" pores which acted as a "barrier". Only after 84 days when degradation of the polymer started, bone ingrowth was enabled into the "liberated" HA pores (Fig. 3b) and better bone ingrowth into the centre of the implant was observed (Fig. 2d).

3.3. Scanning electron microscopy

Scanning electron microscopy of the pure HA cylinders showed the open, foam-like trabecular macrostructure of Endobon® with its interconnecting macro- and micropore system (Fig. 4a). The glycerol-L-lactide coating polymer was found on the ceramic itself. However, this coating of the ceramic filled the HA pores and often led to "locking" of the pore system.

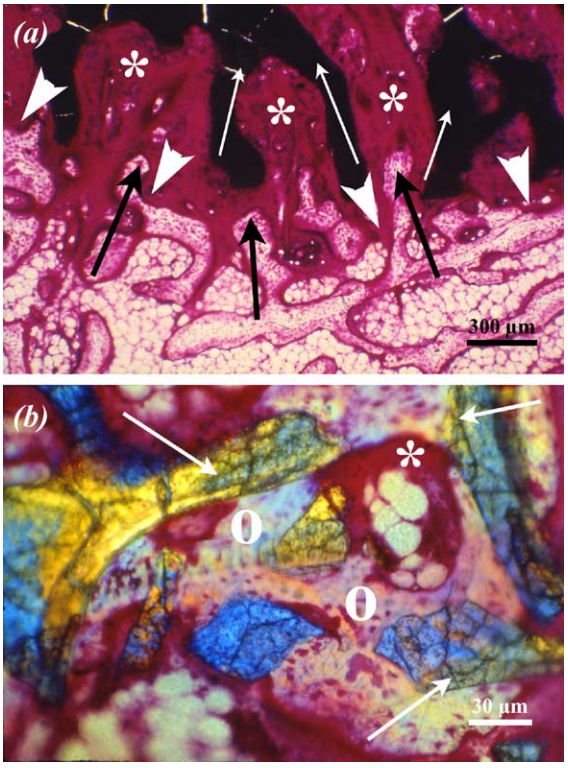

Fig. 3. Basic fuchsin histology showed that the surrounding host bone tried to grow into the glycerol-L-lactide-coated HA implant (black arrows) after 42 days (a). But the polymer (*) which filled the pores of the ceramic (white arrows) appeared as a barrier for the osteoblasts and, therefore, no bone ingrowth was possible. After degradation of the polymer (*) after 84 days fluorescence staining revealed that the pores of the HA (white arrows) were opened and new bone formation and bone ingrowth into the pores could be detected (o).

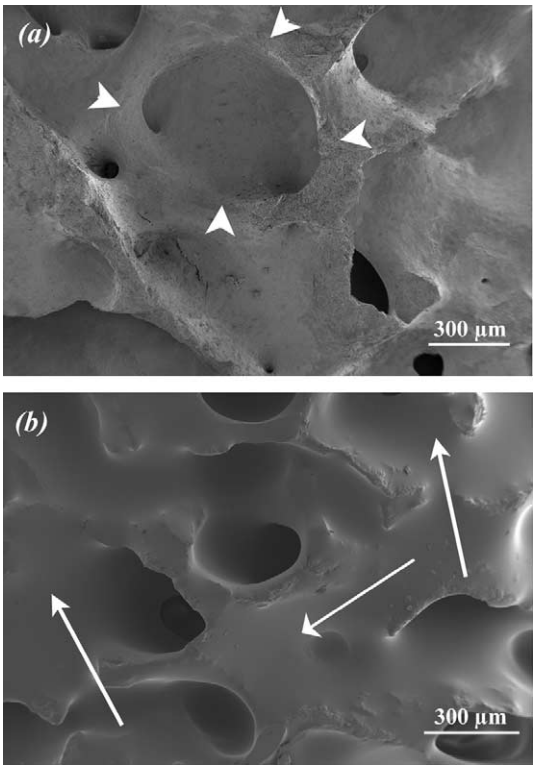

Fig. 4. Also scanning electron microscopy showed that the normally open pores of Endobon® (arrowheads, (a)) are "locked" by the coating polymer (white arrows, (b)).

4. Discussion

Polymers can be used for the coating of hydroxyapatite (HA) and vice versa. In the first case, the osteoconductive properties of the HA is used to improve the osteointegration of the coated polymer device [17], e.g. for HA-coated-poly(L-lactide) composites [18]. For the latter case, an improvement of osteoblast adhesion on HA-titania-poly(lactide-co-glycolide) sol-gel titanium implants was recently shown [19]. Furthermore, composites of HA with poly(DL-lactide) were found to exhibit favorable release kinetics for the local delivery of gentamicin for bone infection treatment [20].

Combinations of polymers with HA and growth factors in form of microspheres [21] or with small HA particles [22] have been shown to enhance new bone formation. However, to the authors' best knowledge there is no published in vivo study trying to improve bone ingrowth into solid HA ceramic implants by the use of a coating polymer.

In a previous work of the authors of the current study improvement of in vitro release kinetics of bFGF-coated HA implants could be shown by the use of glycerol-L-lactide as coating polymer with a more homogenous and a more sustainable release of the growth factor of 20 days compared to only 4 days in polymer-free HA implants [15]. However, there was a significant delay in bone ingrowth in bFGF-coated HA implants in which glycerol-L-lactide was used compared to pure bFGF-coated HA cylinders without the coating polymer [15]. This phenomenon was most likely attributable to "locking" of HA pores by the polymer but inactivation of bFGF by the solvent acetone was also possible. This study was done to differentiate between these two potential reasons because no growth factor was used and, therefore, inactivation of the growth factor by acetone could be excluded.

If "locking" of the HA pores was responsible for the delay, this glycerol-L-lactide technique has to be considered to be unsuitable for coating of HA implants with growth factors. If the delay was not attributable to the polymer itself but to acetone, glycerol-L-lactide would remain of interest in the field of HA implant coating.

The current study revealed that new bone formation and bone ingrowth was significantly delayed in the glycerol-L-lactide-coated HA implants compared to pure HA both after 42 and 84 days. "Locking" of the HA pores by the glycerol-L-lactide polymer due to its slow degradation is the reason for this negative effect on bone ingrowth. Histology clearly showed that the surrounding host bone was "activated" and tried to grow into the implant, but the pores were almost completely blocked by the coating polymer. The filling of the HA pores was also clearly visible in scanning electron microscopy. The phenomenon of osteoconductivity is responsible for bone ingrowth in HA ceramics which is mainly related to pore morphology (pore connectivity and percent porosity) [23] and pore size of >100 μm [24,25]. The locking of the pores by the polymer obviously "neutralized" the osteoconductive properties of Endobon® and prevented the surrounding host bone from ingrowth into the implant.

The used HA ceramic Endobon® of the current study has already shown complete bone ingrowth after 84 days in another study using the same animal model [14]. These results are comparable to the findings of the current study in which also after almost complete bone ingrowth in the uncoated HA implants was found (92%). There was a delay in bone ingrowth between the glycerol-L-lactide-coated implants and the pure HA ceramics of about 5 weeks in this study.

Glycerol-L-lactide-coating plus bFGF has led to new bone formation of 38% after 84 days in another study of the current authors [15] compared to 28% in the same HA implants with the coating polymer but without bFGF. This observation underscores the positive effect of bFGF on new bone formation and bone ingrowth also for the implants with the coating polymer.

Due to the fact that no growth factor was included in the current study the delay in bone ingrowth cannot be shifted to inactivation of the growth factor by acetone which was used as solvent for the coating polymer. Also other authors used acetone as solvent for coating procedures but never reported on inactivation of the growth factor [26–28].

It can be concluded that mechanical "locking" HA pores by the glycerol-L-lactide coating polymer due to its late degradation was responsible for the significant delay in bone ingrowth in the current and in a previously conducted study [15] and, therefore, this coating technique is unsuitable for delivery of growth factors from HA ceramic implants.

A new coating polymer to improve release kinetics of bioactive molecules and bone ingrowth for HA implants must exhibit physiochemical properties that do not interfere significantly with the crucial osteoconductive properties of the HA itself like pore morphology and pore size. This limits the use of relatively thick layers or requires the use of fast in vivo degrading polymers which might on the other side limit the improvement of release kinetics.

The current study clearly showed that in vitro findings on improved release kinetics are not sufficient to prove enhanced bone ingrowth. In vivo testing of bone ingrowth of polymer-coated implants

without any bioactive substances help to test the pure effect of the polymer itself on bone ingrowth of the coated implant and should precede further in vivo investigations.

5. Conclusion

A previously conducted in vitro study showed that glycerol-L-lactide improved release kinetics of bFGF from bFGF-polymer-coated implants. However, the current study revealed that the use of glycerol-L-lactide as coating polymer for delivery of growth factors from HA implants led to significant bone ingrowth delay. This phenomenon is attributable to the "locking" of the HA pores by the polymer. Only after starting degradation of the polymer within the implants the surrounding host bone is able to grow into the implant.

Therefore, it can be concluded that glycerol-L-lactide improves in vitro release kinetics but significantly delays bone ingrowth of bFGF-glycerol-L-lactide-coated HA implants.

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