Release of Gentamicin From Bone Regenerative Materials: An *In Vitro* Study

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Abstract: Antibiotic loading of bone regenerative materials is a promising way to protect augmentation procedures from infection during the resorption phase of bone substitutes. Especially in the early stage of implantation, it should protect the grafted site against microbiological pathogens. The present study reports the release kinetics of gentamicin after loading from two synthetic bone filling materials. The first, BONITmatrix® , is a biphasic calcium phosphate silica composite obtained by the sol-gel route consisting of 13% silicon dioxide (w/w) and calcium phosphates (hydroxyapatite/β-tricalcium phosphate 60/40 w/w). The second, Synthacer®, is a sintered hydroxyapatite ceramic. Gentamicin was loaded by dipping and by vacuum coating. Release kinetics of the loaded Gentamicin was investigated by fluorescence polarization immunoassay and by staphylococcus aureus assay. By dipping, loading failed for Synthacer, and it was 12.7 mg gentamicin per gram bone substitute for BONITmatrix. By vacuum coating, loading was 11.3 mg gentamicin per gram bone substitute for Synthacer and 7.4 mg gentamicin per gram bone substitute for BONITmatrix. Distinct release kinetics were measured. For Synthacer, a high initial release was followed by a lower protracted release level up to 28 days. For BONITmatrix release was continuous over the investigated 70-day period. The present data suggest that the porosity properties at the nano- and microscopic levels, or the composition are responsible for antibiotic loading and subsequent release. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 81B: 23-29, 2007

Keywords: bone regenerative material; antibiotics; calcium phosphate; silica; hydroxy apatite; release kinetics

INTRODUCTION

Orthopaedic procedures are highly predictable on the long-term.[1] Nevertheless, the infection risk still represents a non-negligible concern in orthopaedic surgery; infection rates in the 0.5-5% range have been reported for total joint arthroplasties.[2,3] Although rather infrequent, the consequences of prosthetic joint infections are marked in terms of morbidity and complications[4]; in addition, they involve high hospitalization costs.[5] Prophylactic use of antibiotics lowers significantly the risk of revision[6] when administrated systemically or locally. This is the reason why PMMA bone cement has been loaded with gentamicin and have become the golden standard for fixation of cemented arthroplasties in Europe.[7] Efficacy of this prophylaxic use led also to the development of implants coated or loaded with antibiotics.[8]

As in any orthopaedic surgery, bone augmentation procedures are also exposed to the risk of infection.[9,10] Blocks and granules made of hydroxyapatite, β-tricalcium phosphate, or calcium sulphate are used as biomaterials; they might be colonized by bacteria and viruses and damage the adjacent tissues.[11] Foreign biofilm formation starts with the attachment of free-floating microorganisms, and it is followed by the formation of colonies adhering to the surface.[12] When the first colonies are not immediately isolated from the surface, they might remain permanently anchored. Bacteria living in a biofilm are known to display different properties than free-floating bacteria.[13] This is due to the dense and protecting environment of the biofilm that allows cooperation between the microorganisms and interaction in various ways.[14] The dense extracellular matrix and the outer cell layer protect the interior colonies; this might result in increased resistance to antibiotics. Furthermore, the human immune system might be also prevented from reaching the bacteria living in the biofilm. Over weeks or months, the slowly growing infectious microorganisms
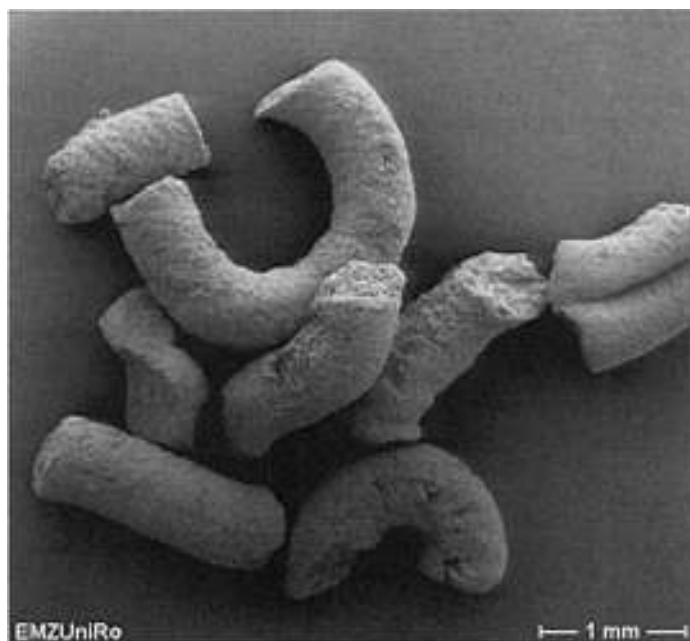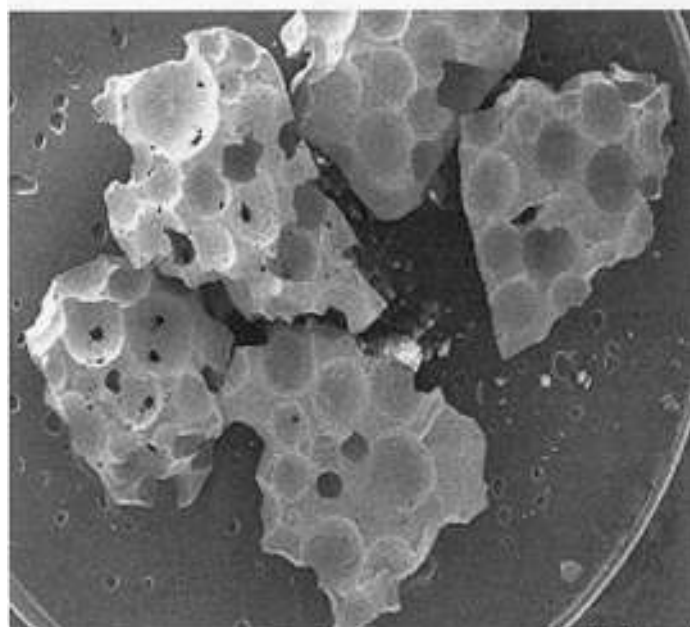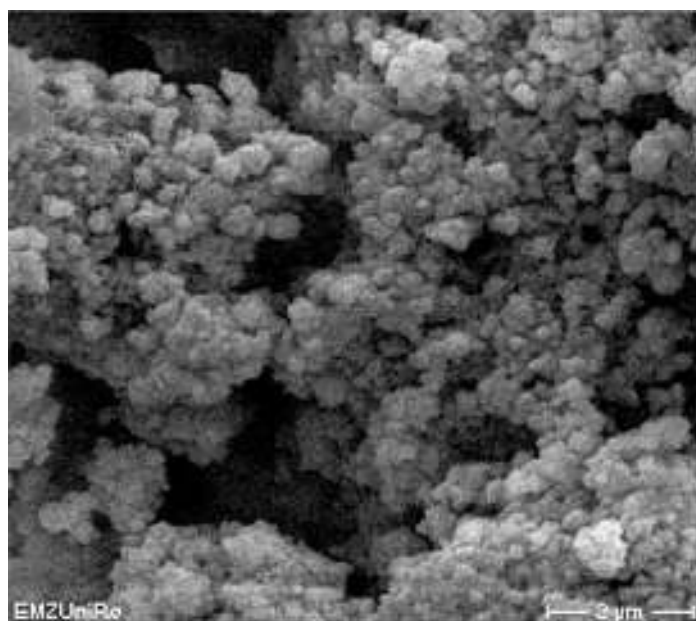
Figure 1. (a) SEM micrograph of BONITmatrix (x50) and (b) SEM micrograph of Synthacer (x20).

remain often undetected in a biofilm layer, until a late postoperative infection occurs.[15]

Coating bone substitute materials with antibiotics should prevent adherence and colonization of bacteria at the biomaterial surface[16-18]; it might be a promising way to avoid postoperative infections. Local antibiotic prophylaxis by loading of filling biomaterials is a more relevant approach than providing systemic antibiotics. In the latter, the active antipathogenic agent floods into the entire body while only a reduced portion of the absorbed antibiotics is available at the target implantation site. This explains why coverage reached by systemic antibiotic therapy is not always efficient enough to protect the surface of bone substitute materials. In addition,

relevance of protracted antibiotic release from the biomaterial surface is high because protection can thus be ensured from surgery on over a longer postoperative period. The protection period may last for a few days up to several weeks; during this time, the coating should prevent infection from extension and should assist the human immune system against pathogens. Furthermore, because antibiotics are water-soluble, they might diffuse from the filled site and protect the surrounding bone from infection.

Antibiotic coatings should be biocompatible and fully resorbable; they should not interfere with the intrinsic osteoconductive properties of the bone filling material and should not alter their bone substitutive potential. Gentami-
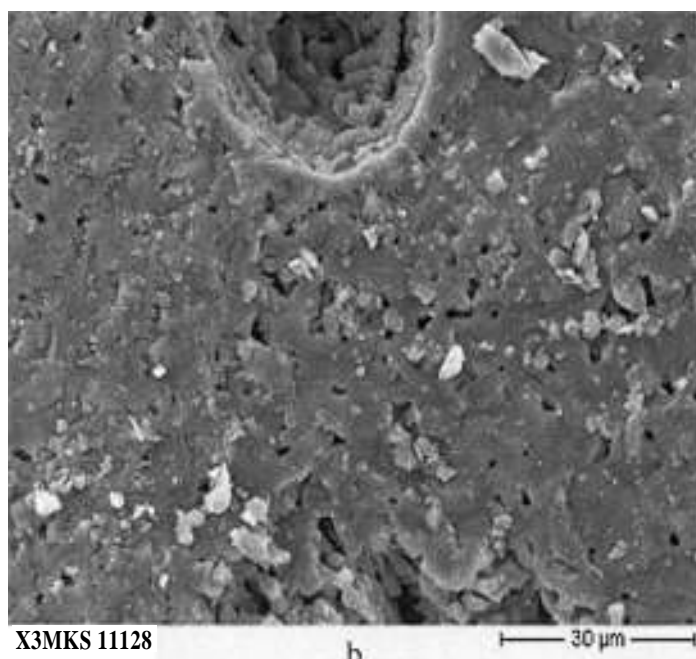
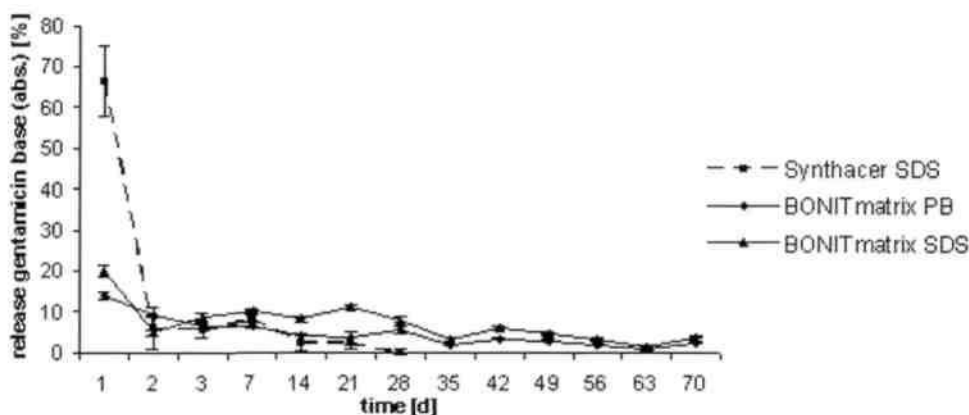Figure 2. (a) SEM micrograph of BONITmatrix (x 10,000) and (b) SEM micrograph of Synthacer (X2000).

Figure 3. Absolute release rates of gentamicin as a function of sampling time measured by the FPIA method. Mean $\pm$ SD is provided ($n = 5$).

cin sulfate belongs to the aminoglycoside antibiotic family. It is efficient against microorganisms that are responsible for bone infection; it has been documented to treat deep bone infections.[19] In addition, it has been extensively used with success in orthopaedics since 1970s, in combination with bone cements.[7,20,21] This is the reason why gentamicin was considered here as a good candidate for antibiotic coating of bone substitute materials.

The aim of this article is to report on the release kinetics of gentamicin from two distinct biomaterials over a period of up to 70 days *in vitro*. The purpose was to get information about their potential for a long-term coverage.

MATERIALS AND METHODS

Materials

BONITmatrix® and Synthacer® were investigated. BONITmatrix (DOT GmbH, Rostock, D) was obtained by the sol-gel route. Granules of BONITmatrix were prepared by adding a biphasic calcium phosphate powder, consisting of hydroxyapatite and β-tricalcium phosphate in a 60/40 weight ratio, to a hydrolyzed tetraethoxysilane solution containing HC1 as a catalyst.[22,23] After gelation, the composite was

formed and dried at 200°C for 2 h. Granules with a mean diameter of 0.6 ± 0.1 mm and 1-4 mm in length were formed [Figures 1(a) and 2(a)]. BONITmatrix is a CE Mark product (class III) that was approved by TUV.

Synthacer (medArtis, Munich, D) is a synthetically manufactured, porous bone substitute material made of hydroxyapatite according to ISO 13779-1; phase purity of this sintered ceramic is higher than 99%.[24] It is available in granules, blocks, and cylinders of various sizes. In the present study, granulate particles in the 2.5-4.8 mm size range were used [Figure 1(b) and 2(b)]. Synthacer is a class IIb product with the associated regulations and product code.

All the samples were 7-sterilized before loading with gentamicin.

Loading of Gentamicin

Loading of the biomaterials were carried out according to two different methods, vacuum coating (SDS process) and dipping (PB process). Gentamicin sulphate was dissolved in a sodium dodecylsulphate solution (weight ratio 1:1) and the biomaterials were coated by spraying and subsequently drying under vacuum condition. Gentamicin loading was 11.3 mg gentamicin per gram bone substitute for Synthacer
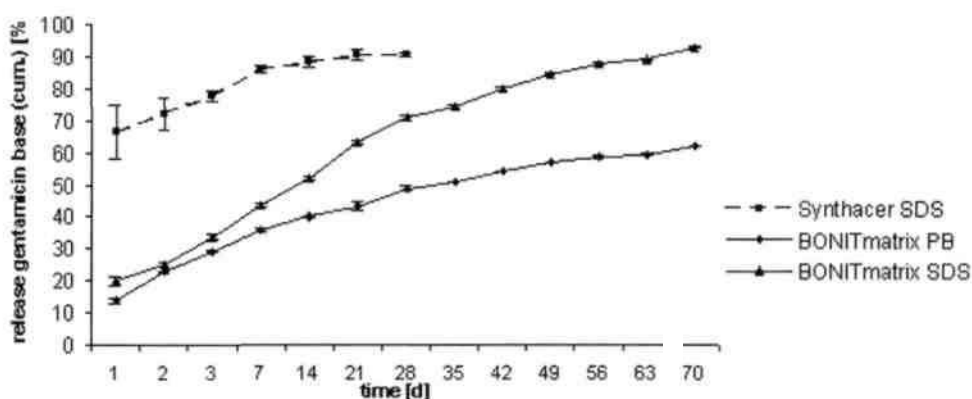

Figure 4. Cumulative release rates of gentamicin as a function of time measured by the FPIA method. Mean $\pm$ SD is given ($n = 5$).
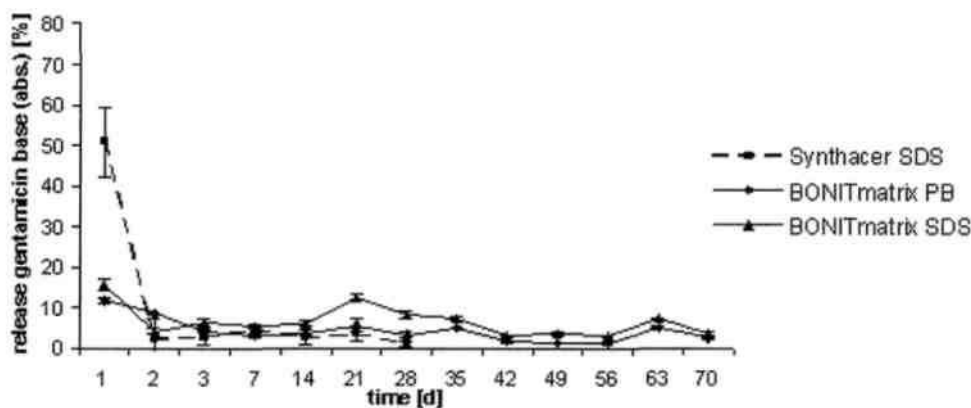
Figure 5. Absolute release rates of gentamicin in function of sampling time measured by the SAS method. Mean $\pm$ SD is given ($n = 5$).

(Synthacer SDS) and 7.4 mg gentamicin per gram bone substitute for BONITmatrix (BONITmatrix SDS). Loading by dipping was performed in a phosphate-buffered solution *(0.05M)* of gentamicin sulphate at room temperature for 24 h; concentration of the solution was 2 mg gentamicin/mL of buffer solution. Loading was 12.7 mg/g for BONITmatrix (BONITmatrix PB), and loading failed for Synthacer. The amount of gentamicin bound to the material was quantified spectrophotometrically after acidic pulping of the material (BONITmatrix) or by extraction of gentamicin using an aqueous extraction solution containing an ion exchanger (Synthacer). In addition, the gentamicin content in the loading solution was also determined by FPIA.

Kinetic Release

Kinetic release measurements were duplicated on five distinct samples per group. One gram of biomaterial was soaked in a solution of 20 mL of phosphate buffer (0.01*M*) pH 7.4 and maintained at 37°C. Sampling was performed after 1, 2, 3, 7, 14, 21, and 28 days; if release was still detected, weekly sampling was continued up to 70 days (Figure 3). Aliquots of 10 mL were sampled; at each interval, 10 mL of freshly prepared phosphate buffer were added after sampling.

Concentration of antibiotics in the solution was measured in parallel by two methods, fluorescence polarization immunoassay (carried out at Heraeus Kulzer; FPIA-Abbott TDx fluorescence polarization immunoassay, Abbott Laboratories, IL) and staphylococcus aureus assay (carried out at DOT; SAS). FPIA was calibrated with a corresponding calibration kit (detection range, 0.5-10 µg/mL). Each test run was performed twice including control samples (concentration =1, 4, and 8 µg/mL*)* to verify the results. Detection of released antibiotic by the agar diffusion test was carried out with the staphylococcus aureus ATCC 29213. Standard nutrient plates (Merck, 1.10416.0001, Darmstadt, D) were used for the agar diffusion test. The release or standard solutions (50 ,µL) were pipetted in punched slots (diameter 5 mm). The negative control showed no inhibited area. The calibration curve (detection range, 5-100 µg/mL) obtained by the staphylococcus aureus assay was mathematically calculated by fitting, with the Origin Pro 7.5 software (OriginLab, Northampton, USA). The diameter of inhibited area for gentamicin base concentration 5 µg/mL was 13 mm and for 100 µg /mL was 22 mm. Samples were collected and stored at 4°C; before investigation, they were warmed up to room temperature. The SAS test was carried out under flowbox conditions.
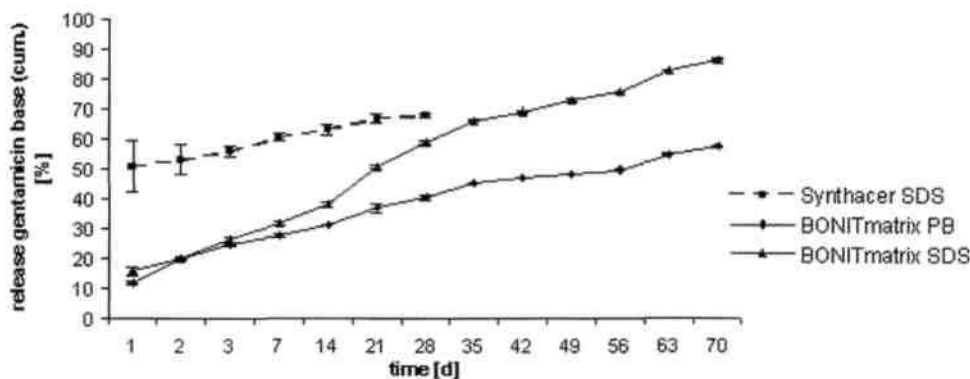

Figure 6. Cumulative release rates of gentamicin in function of sampling time measured by the SAS method. Mean $\pm$ SD is given ($n = 5$).
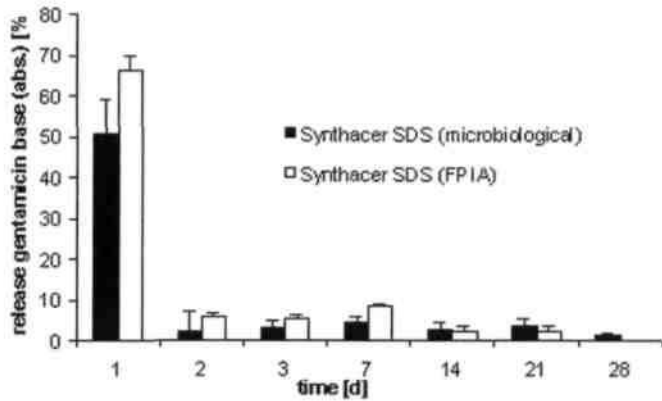
Figure 7. Gentamicin release from Synthacer loaded by SDS and measured by the FPIA and SAS methods. Release is given in absolute release percentage.

RESULTS

Gentamicin release was measured by the two methods. Cumulative and absolute release rates of gentamicin obtained by the FPIA are reported for all groups on Figures 3 and 4. Figures 5 and 6 show the results obtained by the SAS method for all three groups. The release rates are graphed in Figures 3-8 as a percentage of total loading of sample. Figures 9 and 10 are based on weight data in the measuring unit "mg." The kinetic curves differed according to the investigated material. Release from Synthacer loaded by the SDS process showed an initial burst corresponding to 60% of the loaded gentamicin after 1 day; this was followed by a low release that lasted up to 28 days as shown in Figure 7. Release of gentamicin from BONITmatrix, either loaded by SDS or by PB, was still detected for a period up to 70 days. Release was continuous with a low initial burst effect. After the first week, a cumulative amount representing 32 and 28% of the loaded gentamicin were released for the SDS and PB loading process, respectively. Release over time was wave-like as shown in Figure 8. For all investigated groups, similar release profiles were obtained by the FPIA and SAS methods.
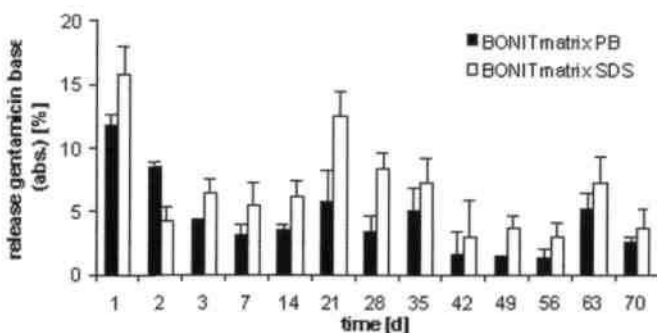

Figure 8. Gentamicin release from BONITmatrix granules loaded by the SDS and PB processes and measured by the SAS method. Note the wave-like aspect of the release.
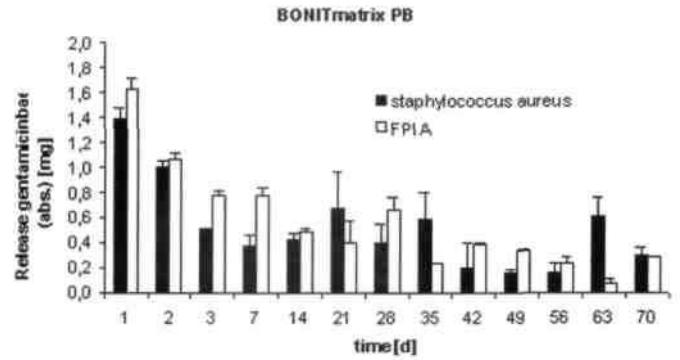

Figure 9. Comparison of the absolute release of gentamicin from BONITmatrix PB according to the detection method.

DISCUSSION

This *in vitro* experiment was undertaken to compare the release of gentamicin from two distinct materials loaded in two different ways. It was aimed to get information on the extension of the antibiotic protective activity when loaded in bone substitutive materials.

Loading method played a key-role; PB loading was inefficient for Synthacer and led to a higher concentration of gentamicin for BONITmatrix than SDS loading. Both methods showed similar release profiles for both bone substitute materials (Figures 9 and 10).

However, for Synthacer and BONITmatrix the release values achieved by FPIA were slightly higher then by SAS (Figures 9 and 10). The reason can be because the FPIA method detects the total amount of gentamicin in the elution fluid, whereas the SAS method detects only the microbiologically active gentamicin.

A comparison between SAS and FPIA detection methods showed a good correlation. Values for the regression coefficients of graphs for the correlation of average results of both detection methods were not significant different (Figures 11 and 12). The regression coefficient for BONITmatrix PB was 0.576 and for BONITmatrix SDS 0.6163. The p values of 0.0026 (BONITmatrix PB) and 0.0013 (BONITmatrix
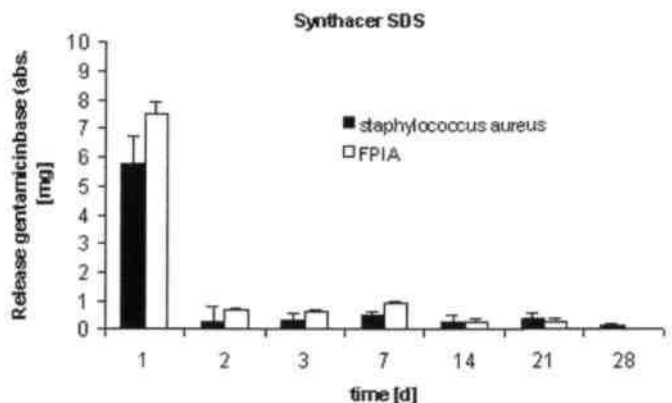

Figure 10. Comparison of the absolute release of gentamicin from Synthacer SDS according to the detection method.
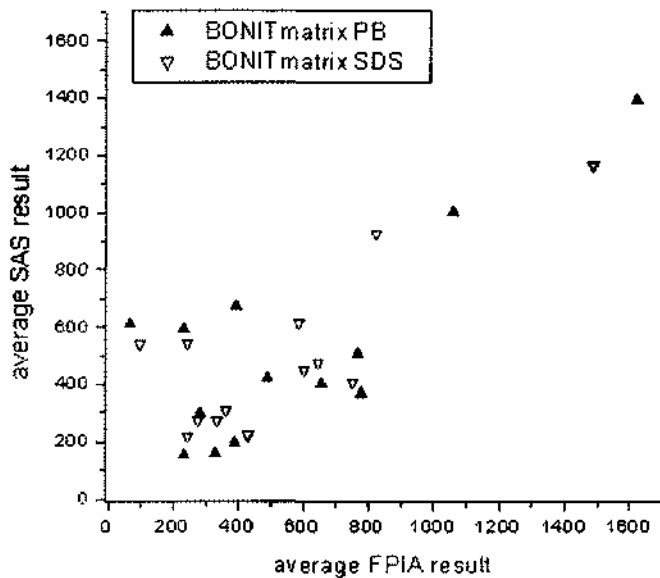
Figure 11. Correlation between values of average SAS results and average FPIA results for BONITmatrix PB and SDS (BONITmatrix PB: $R^2 = 0.576$; $y = 0.6358x + 164.98$; $p = 0.0026$ ($n = 13$); BONITmatrix SDS: $R^2 = 0.6163$; $y = 0.6127x + 170.68$; $p = 0.0014$ ($n = 13$)). Average measuring results are given in $\mu g/g$.

SDS) reflect a high probability of correlation between the two used measuring methods for gentamicin.

Synthacer showed in this connection a higher regression coefficient of 0.9922. A possible interpretation is that we found 1 relatively higher value in comparison with 6 smaller values in a tight spreading pattern (Figure 10), respectively.

Synthacer has a porous structure similar to cancellous bone with micro- and macroopenings in the shell structure[24,25] [Figure 2(b)]. The diameter of the pores can reach several hundreds micrometers and allow bone for ingrowth and integration in the newly formed bone. Usually, bone substitutes coated with pure gentamicin sulphate release the total amount of antibiotics during the first day without retarding release effect.[26] Therefore, the postoperative antibiotic protection that can be expected for this type of dense material with simple gentamicin sulphate coating is very short.

The retarding release profile obtained here for Synthacer was ensured by sodium dodecylsulphate,[26] used here as an additive. Synthacer showed a high initial release of about 60% of the total amount during the first day. This high initial antibiotic burst was followed by a protracted release at a lower but still microbiologically efficient level for up to 4 weeks. From day 21 to day 28 still ~135 μg gentamicin per gram of bone substitute material were released, and gentamicin concentration in the eluent was higher than the minimum inhibitory concentration against staphylococcus aureus, one of the most relevant bacteria causing bone infections (Figure 10). After 28 days, no further gentamicin was eluted (Figures 4 and 6).

The release profile for BONITmatrix was different. No initial burst was evidenced; release was detected over a period of 70 days. Over a period from day 7 to day 35 an

absolute release rate per week of 492 μg gentamicin per g BONITmatrix PB was detected. Bone substitute material (169 μg gentamicin per g bone substitute material) was eluated from day 42 to day 56. After the following week the release rate increased to 611 $\mu g/g$ and then from day 63 to day 70 the release decreased to 297 $\mu g/g$ (Figure 9). After 63 days, a break-up of the material was noticed. The reason for this lengthy release profile might be due to the structure properties of the composite produced by the sol-gel route. It is composed by a silicon dioxide network (13%) that contains embedded calcium phosphate particles (87%) into a nanoporous composite where porosity is 60% $\pm$ 5% for a density of 0.90 $\pm$ 0.05g/cm^3 [27] [Figure 2(a)]. The surface area is high and reaches 90 m²/g; the internal pore size distribution shows a high percentage of nanopores in the 20–80 nm range. [27] The existing nanoporosity of this composite material permits a complete diffusion of the biological fluids. The interconnecting pore system thereby creates the conditions for high capillarity and high adsorptive properties. These two properties have been confirmed when immersed in blood.[28]

It should be noted that, for BONITmatrix, the absolute release values did not decrease monotonically. Both the FPIA and SAS methods showed a wave-like aspect of the release profile. This might be attributed to the formation of equilibrium phases in the diffusion process of gentamicin. Noncontinuous sampling in a 24 h frequency would have allowed for adsorption/desorption processes at the internal and external surface of BONITmatrix. The antibiotic fixation to the composite may have also influenced the over-all release.[27] Gentamicin is a biochemical compound with functional groups, which might interact with calcium or phosphate ions.

In conclusion, gentamicin loading was obtained for both bone substitute materials. They might be protected against microbial adhesion and proliferation by an appropriate
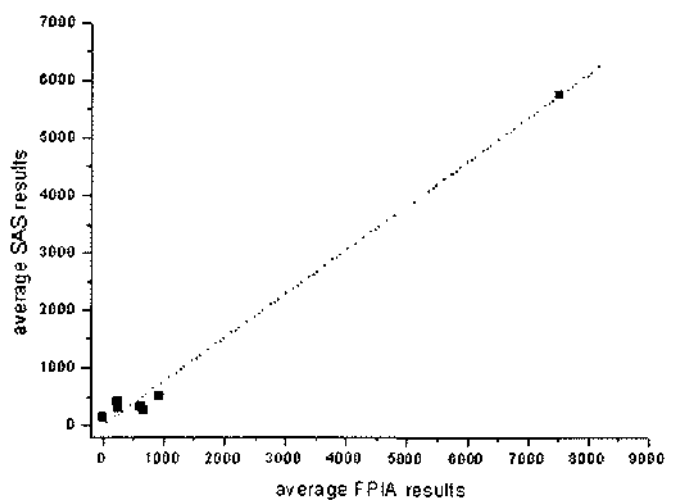

Figure 12. Correlation between values of average SAS results and average FPIA results for release of gentamicin from Synthacer SDS ($R^2 = 0.9922$; $y = 0.7626x - 0.017$; $p < 0.0001$ ($N = 7$)). Average measuring results are given in $\mu g/g$.

coating. Depending on the microstructure or the chemical composition, different release profiles up to 28 days or up to 70 days can be obtained in *in vitro* release studies. This can lead to a protracted local action of the antibiotic. Further animal and then clinical studies should be completed to confirm the effectiveness of either of these materials as carrier for gentamicin.

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