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Biphasic mode of antibacterial action of aminoglycoside antibiotics-loaded elastic hydroxyapatite–glucan composite

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ABSTRACT

Following the quest for new composite materials for bone tissue engineering, a novel elastic hydroxyapatite–glucan composite loaded with two aminoglycoside antibiotics was prepared. The porosity of the composite and the drug release profiles in closed-loop and semi-open systems were tested. The antibacterial activity of the drug was estimated against two Gram-positive and two Gram-negative bacterial strains causing orthopedic infections. It was found that the loaded antibiotic acted in a biphasic mode. The majority of the drug was released within 48–119 h in a pore-dependent manner and inhibited the bacterial growth in the culture medium. However, a small residual amount of the drug was bound to the composite microstructure via ionic interactions and acted as a short-lived barrier against bacterial adhesion to the composite, although the surrounding medium was no longer protected against bacterial infection. Sub-inhibitory concentrations of the released drug were observed in the medium only during the last two days of the experiment (minimized risk of occurrence of drug-resistant strains). Thus the novel drug-loaded elastic hydroxyapatite–glucan composite, demonstrating a biphasic mode of antibacterial action, may be recommended for antibiotic prophylaxis in bone substitute implantation, with less emphasis on the treatment of bone infections.

1. Introduction

Hydroxyapatite (HAp; $Ca_{10}(PO_4)_6(OH)_2$), an equivalent of the main mineral component of bones and teeth, is extensively used in the form of granules and porous scaffolds as an artificial bone substitute in regenerative medicine. It is appreciated for its natural bone-like structure, bioactivity, biocompatibility, osteoconductivity and the lack of immunogenicity and toxicity. Moreover, many bone-mimicking composites described in the literature are based on HAp ceramics as their main component (Krisanapiboon and Buranapanitkit, 2006; Boanini et al., 2006; Todo et al., 2006).

HAp ceramics present many advantageous properties, but also some drawbacks. One of the problems concerning HAp ceramics is its poor elasticity and a substantially high Young's modulus which is reduced in natural bone tissue due to the presence of collagen fibers. In artificial composites, this role is played by polymers increasing elasticity, such as PLLA (Todo et al., 2006), collagen isolated from animal tissues (Lawson and Czernuszka, 1998) or chitosan (Kong et al., 2006; Oliveira et al., 2006). The use of pure HAp ceramics for surgical purposes is limited due to its relatively poor convenience during orthopedic procedures (low surgical handiness). Therefore, observed may be a tendency to fabricate biomaterials which combine the benefits of calcium phosphate ceramics with a level of plasticity/elasticity convenient for surgeons. Some ceramics-based composites offering these properties have appeared on the market (EasyGraft™ by Degradable Solutions SA, Switzerland, Plexur M and P by Osteotech, USA and Cerapatite-Collagen by Ceraver Osteal, France); some have been reportedly subjected to laboratory and clinical tests (Chen et al., 1998; Song et al., 2009). However, extensive studies are still being performed in this field.

Hydroxyapatite ceramics surface is also known as a possible target for the adhesion of bacteria and fungi (Verret et al., 2005; Clark et al., 1985; Aronov et al., 2007). After the implantation, it may be colonized by numerous bacteria species, thus becoming an infection center (You et al., 2003; Jordan et al., 1999) and increasing the possibility of development of a highly resistant bacterial biofilm. Among the bacteria responsible for these infections, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Gram-positive), as well as *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative) are the most frequently observed species (Sanderson, 1991; Goel, 2006). Implant-related, mostly nosocomial infections in orthopedic surgery are a serious problem and result in a high mortality rate, observed in up to 2.2% surgeries involving hip arthroplasty

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(Pedersen et al., 2010; Pichavant et al., 2012) and even 3–4% surgeries involving total joint arthroplasty (revision hip surgery) (Goel, 2006). Systemic antibiotic treatment is a standard protocol in such cases. However, in the case of the poorly vascularized bone tissue, the concentration of such administered antibiotic must be very high to be effective. This increases the probability of drug-related toxicity. Therefore, to minimize the risk of antibiotic side effects (e.g., ototoxicity or nephrotoxicity), as well as the occurrence of bacterial biofilm and drug-resistant strains, local antibiotic treatment seems to be the optimal therapy. Antibiotic-loaded hydroxyapatite ceramics or ceramics-based composites are therefore frequently used as fillers of bone defects for the prevention and treatment of prosthesis-related infections (Shinto et al., 1992; Krisanapiboon and Buranapanitkit, 2006; Ginebra et al., 2006; Chai et al., 2007; Laurent et al., 2008). This allows clinicians to overcome the disadvantages of systemic drug delivery and to avoid the risk of reoperation for the removal of an infected implant.

Most studies concerning the efficacy of medicament-loaded composites focus on the antibacterial action of a soluble drug, eluted into the medium surrounding the material. However, the estimation of the residual antibacterial activity, remaining in the composites after the release of the soluble drug, is rarely reported. Even a small amount of residual antibiotic, entrapped or adsorbed to the biomaterial via specific and nonspecific interactions, may protect the implant against bacterial adhesion even if the drug is no longer released into the surrounding medium. In such case, the residual drug may be considered as a weakly immobilized drug, preventing the adhesion of bacterial cells directly to the biomaterial and the formation of a highly resistant biofilm. Therefore, attention should be focused not only on the activity of the released antibiotic, but also on that of the residual antibiotic in the drug-loaded composite, to fully explain the mechanism of its bactericidal action.

In our recent paper, we described the synthesis and properties of a novel elastic HAp–glucan composite, elaborated based on the HAp granules of a particularly high porosity (Belcarz et al., 2013). The aim of this study was to investigate this elastic composite in its modified version, as an antibiotic-loaded carrier for local drug delivery. The composite was designed to exhibit a moderate rate of drug release and to minimize the risk of development of drug resistance in bacteria. For this purpose, aminoglycoside antibiotics (gentamicin or amikacin sulfate) were selected as suitable models because of their wide spectrum of antibacterial activity which enabled their common use in preventing infections in orthopedic surgery. The composite was loaded with drugs via two methods. The experiment designed for the evaluation of the antimicrobial activity of the fabricated composites included two stages. The first stage involved the estimation of bacterial growth inhibition in the medium surrounding the composite, due to the activity of the released drug. Subsequently, the remaining (residual) antibacterial activity preventing the adhesion of bacterial cells to the composite was established (second stage). This two-stage experiment allowed us to evaluate the complex antimicrobial activity of the antibiotic-loaded HAp–glucan composites and the chemical nature of drug–composite interactions. Four Gram-positive and Gram-negative bacterial strains incriminated in the most common bone implant-related infections were selected as model microorganisms.

2. Materials and methods

2.1. Preparation of samples

Pure composite was prepared as described previously (Belcarz et al., 2013). Briefly, high molecular β-1,3-glucan was mixed with highly porous HAp granules (67% open porosity; fraction size: 25 wt%: 0.2–0.3 mm and 75 wt%: 0.5–0.6 mm) at an appropriate weight ratio (17:83), baked at 100 °C for 10 min and finally dried at 25 °C for 24 h. β-1,3-Glucan from *Alcaligenes faecalis* (DP 450) was supplied by Wako Chemicals, Japan. HAp granules were prepared at the Faculty of Materials Science and Ceramics, AGH University of Science and Technology in Cracow, Poland.

Amikacin sulphate (250 mg/ml; Bioton, Poland) and gentamicin sulphate (40 mg/ml; KRKA, Slovenia), used as model antibiotics, were loaded into the composite via 2 methods, as described in the patent specification (Belcarz et al., 2009a). Briefly:

Method M1 (loading before composite preparation): prior to the composite preparation, the drug solution at an appropriate concentration in ultrapure water was loaded into dry HAp granules under low pressure until it was completely absorbed. The volume of the drug solution was optimized to ensure a uniform drug distribution within the granules. Subsequently, the granules were dried at 20 °C and combined with β-1,3-glucan, while the elastic composite was prepared as described above.

Method M2 (loading after composite preparation): drug solution at an appropriate concentration in ultrapure water was loaded into a dried composite sample under low pressure until it was completely absorbed. The volume of the drug solution was calculated based on the sorption index to ensure an optimally uniform drug distribution. Finally, the composites were dried at 20 °C.

2.2. FTIR analysis

The FTIR spectra of pure antibiotics, the HAp–glucan composite and the antibiotics-loaded composites were obtained using an IR spectrometer (Vertex 70, Bruker, USA) in ATR mode (diamond crystal), 64 scans with 4 cm^{-1} resolution, at a wavelength range of 370–4000 nm.

2.3. Porosity

The open porosity and pore size distribution of highly porous HAp granules and samples of the composite were evaluated via mercury intrusion porosimetry (MIP) using the AutoPore 9500 Porosimeter (Micromeritics).

2.4. Evaluation of drug release in a closed-loop system

For the evaluation of the parameters of antibiotics release, a flow-through dissolution procedure in a closed-loop system was used. The experiments were performed in the CE4 Sotax apparatus (Donau Lab, Switzerland) using 0.25 g samples of the drug-loaded composite. 50 ml PBS pH 7.4 was used as an elution medium with laminar flow rate (1 ml/min) at 37 °C. 3 ml samples were collected for the estimation of drug concentration at defined time intervals; the system was supplemented with the same volume of fresh PBS to maintain the initial medium volume. Cumulative drug concentrations were calculated based on the results of 4 independent experiments, each in triplicate. For the determination of drug release mechanisms, the Korsmeyer–Peppas model was used with a general equation:

$$\frac{M_t}{M_\infty} = kt^n$$

where M_t is the amount of drug released from the composite in time t, M_∞ the accumulated released drug amount at time $t \to \infty$, k the kinetic constant and n the release exponent.

The interpretation of the drug release mechanism in the case of particular samples was performed via nonlinear regression analysis using the Statistica 10 software.

2.5. Evaluation of antibiotic concentration

The concentrations of gentamicin and amikacin sulphate in solution were estimated according to the method described in The British Pharmacopeia (Pharmacopoeia, 1999) using an HPLC procedure (Gilson HPLC system, USA) or via spectrophotometric assays, as described elsewhere (Ginalska et al., 2004, 2007). The calculations were performed using standard curves for these antibiotics (Fluka, Switzerland; IBA, Poland).

2.6. Antibacterial activity of drug-loaded composites in the semi-open system

A scheme illustrating the experiment schedule for each bacterial strain (with the number of sample pieces and the length of the experimental period indicated for each stage) is briefly presented in Fig. 1 and described in details below.

2.6.1. Stage 1: released drug (activity and quantity) in media

2.6.1.1. Microbiological assay. The following reference bacterial strains were used in the experiment: *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *E. coli* ATCC 25992, *P. aeruginosa* ATCC 27853. The minimum inhibitory concentrations (MICs) of gentamicin against the tested strains were estimated using a standard macrobroth dilution test. The strains were stored in microbanks at −20 °C according to good microbiological practice. Prior to the experiments, the bacteria were cultured on a slant culture medium and then in Mueller–Hinton (MH) broth at 37 °C for 20–24 h. The inoculate was prepared by diluting the latter culture to an appropriate cell density.

Gentamicin-loaded composites containing 1% or 2% drug ($GC_{1\%;M2}$ and $GC_{2\%;M2}$, respectively) were selected for the evaluation of antibacterial activity in a semi-open system. 0.5 g samples of the composites were sterilized with ethylene oxide in paper/plastic peel pouches (1 h at 55 °C, followed by 20 h aeration). Sterile samples were placed in Pyrex tubes with 5 ml Mueller–Hinton broth (Fluka, Switzerland), inoculated with 1.5×10^5 bacterial cells and incubated at 37 °C under stationary conditions. A daily exchange of 60% medium volume was performed under sterile conditions and the cultures were supplemented with fresh bacterial inoculate. The presence of viable bacteria was determined in the collected medium samples using the PhoenixSpec nephelometer (Becton, Dickinson and Company) and by seeding 10 μl of samples on fresh Mueller–Hinton agar (Fluka, Switzerland) plates. Pure composite (PC) was used in the same experiment as a drug-free control.

This stage of the experiment was performed until bacterial growth in the medium became detectable using the nephelometer or measurable on plates.

2.6.1.2. Released drug assay. Simultaneously, the $GC_{1\%;M2}$ and $GC_{2\%;M2}$ composites were tested for the purpose of determination of the quantity of drug release under the same conditions (with 60% medium exchanged daily), with the exception of PBS pH 7.4 used as a medium and the omission of the inoculation steps. In this assay, the protein-rich Mueller–Hinton broth was replaced with PBS due to the limitations of the methods of quantitative gentamicin estimation, based on drug derivatization via amino groups which are present not only in gentamicin (aminoglycoside), but also in proteins. The collected PBS samples were evaluated for gentamicin concentration.

2.6.2. Stage 2: residual drug (activity and content) in composite pieces

This stage was a continuation of Stage 1. It was performed to estimate the possible content of the residual amount of gentamicin, not eluted from the composites during Stage 1. Such a residual drug could prevent direct bacterial adhesion to the composite which was no longer able to inhibit bacterial growth in the surrounding medium. This stage of the test was limited to the control (PC) and $GC_{2\%;M2}$ samples.

2.6.2.1. Microbiological assay. After the occurrence of bacterial growth in Mueller–Hinton broth, the samples were further incubated for 10 days in the same medium, 60% of which was exchanged, to support the bacterial metabolism and growth. The concentration of bacterial cells in the broth was maintained within the range of $1.2–3.0 \times 10^9$ CFU (colony forming units) per ml throughout the experiment. The composite samples were withdrawn from the culture broth at defined time intervals (1, 2, 3, 4, 6, 8, 10 days; 5 samples per interval), carefully washed twice in 50 ml sterile PBS pH 7.4 to remove all the nonadhered bacteria, and stored in 2.5 ml PBS pH 7.4 supplemented with 5% glycerol at 20 °C until the successive step (quantitative and microscopic evaluation of the adhered bacteria).

Subsequently, 5 composite samples collected at each time interval were unfrozen and divided into 2 groups:

- samples from each time interval were subjected to the quantitative evaluation of the adhered bacteria as follows: the samples were ground together with the glycerol–PBS storage solution until a uniform suspension was obtained. Serial dilutions of the suspension were spread onto MH agar plates in aliquots of 50 μl and incubated at 37 °C for 20–24 h. The number of CFU was counted on each agar plate.
- samples from each time interval were subjected to the microscopic evaluation of the adhered bacteria. Scanning confocal laser microscopy (Olympus FluoViev FV1000; Germany) in conjunction with the fluorescence staining technique (incubation with Live/Dead® BacLight™ Kit (Invitrogen, USA), according to the manufacturer's instruction) were used to visualize the bacteria present on the analyzed surfaces. The resolution of 2D laser scan images was 2048 × 2048 pixels. The Imaris Bitplane (Zurich, Switzerland) software was used to render the images of the surfaces.

2.6.2.2. Residual drug assay. The residual gentamicin content in the samples, no longer able to inhibit the bacterial growth in the culture broth, was estimated in an analogous experiment with the exception of PBS pH 7.4 used instead of Mueller–Hinton broth. The samples (6 per interval) were withdrawn from PBS at defined time intervals (1, 2, 3, 4, 6, 8, 10 days) and disintegrated using a mortar with

- 2.5 ml 2 M NaCl (3 samples),
- ultrapure water (3 samples)

to obtain uniform suspensions. After 1 h incubation at 37 °C, the suspensions were centrifuged for 3 min at 12,000 rpm (Sigma 1-14, USA) and the supernatants containing the released drug were estimated for gentamicin concentration.

3. Results

3.1. Drug loading, porosity and FTIR spectra

An elastic HAp–glucan composite was loaded with two antibiotics, gentamicin and amikacin. The composition of the tested samples is summarized in Table 1. It was found that the process of drug loading did not affect the elastic properties of the composite (data not shown). Both drugs are highly hydrophilic aminoglycoside compounds, exhibiting a high solubility in aqueous media.

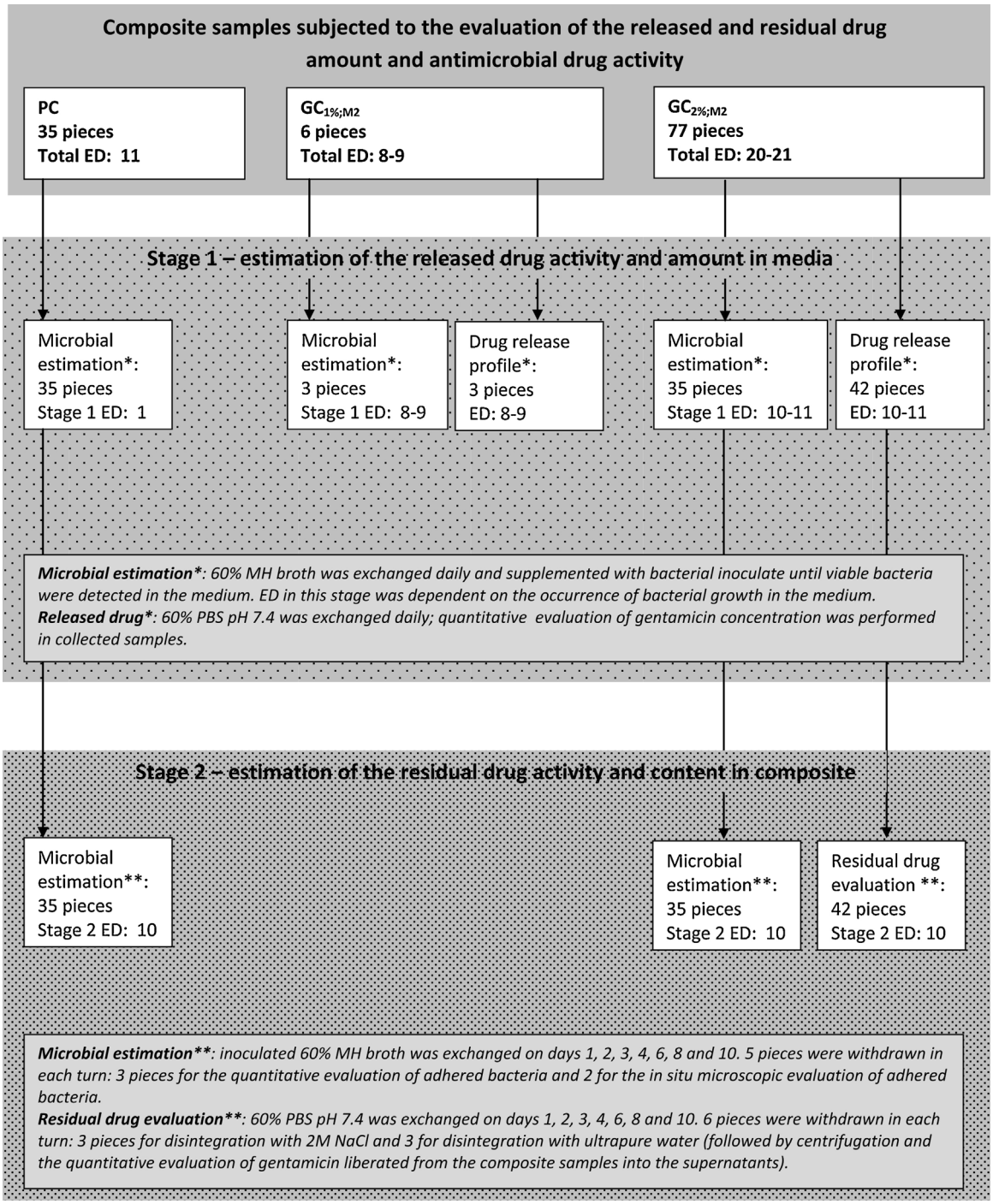


Fig. 1. Scheme illustrating the schedule of the experiment concerning the estimation of antibacterial activity and drug release/content, presenting the number of composite pieces and the duration of each experimental stage. PC, pure composite; $GC_{1\%;M2}$, 1% gentamicin-loaded composite; $GC_{2\%;M2}$, 2% gentamicin-loaded composite. The experiment was performed separately for each bacterial strain. ED, experiment duration (in days).

Table 1
Composition of the tested samples.

Sample code	Antibiotic added to the composite	Antibiotic dose (% of dry composite weight)	Drug loading method
PC (control)	–	–	–
$AC_{1\%;M1}$	Amikacin	1	M1
$AC_{1\%;M2}$	Amikacin	1	M2
$GC_{1\%;M1}$	Gentamicin	1	M1
$GC_{1\%;M2}$	Gentamicin	1	M2
$GC_{2\%;M2}$	Gentamicin	2	M2
$GC_{0.5\%;M2}$	Gentamicin	0.5	M2

M1, granules soaked in the antibiotic solution and dried before the composite preparation; M2, dry ready-to-use composite (PC) soaked in the antibiotic solution and subsequently dried.

This feature is advantageous for the loading methods as it enables manipulation with different doses of drug loaded into the composite. Moreover, it ensures a high uniformity of drug distribution within the composite loaded with antibiotic after its preparation. Drug loading, according to the M1 method, requires more manipulations for the composite synthesis than method M2. The latter, which involves only simple soaking of an already fabricated composite in a sterile aqueous drug solution, may be performed by a surgeon directly before implantation. However, it remained open whether the mode of loading affects the profile of drug release from the composite. Therefore, this aspect was evaluated in further experiments described in this study.

The mechanism of drug release from the composites depends – among other properties – on their porosity. Therefore, this parameter was estimated for the ceramic HAp granules and the elastic composite. The composite exhibited ~67% open porosity and demonstrated a bimodal pore size distribution (Fig. 2a). The peak situated at the higher pore size values (with the maxima of approx. 2.0–8.0 μm) is connected with the existence of pores placed between the HAp granules and the polymeric phase, whereas the peak visible at the lower values (with the maxima of approx. 0.04–0.15 μm) may be assigned to the pores in the HAp granules (Fig. 2b). The ceramic granules exhibited ~63% open porosity.

The FT-IR spectra of the antibiotics and the drug-loaded HAp–glucan composites are presented in Fig. 3. The presence of HAp in the drug-loaded composites was clearly demonstrated by the bands: 963 cm^{-1}, 1022 cm^{-1}, 1088 cm^{-1}, 562 cm^{-1} and 599 cm^{-1} (PO_4^{3-}), and 630 cm^{-1} (OH^-). The bands attributed to the β-1,3-glucan characteristics – 1157 cm^{-1} and 888 cm^{-1} (C_1—O—C_3 stretching vibration) – were significantly flattened both in the spectra of the amikacin-loaded and gentamicin-loaded composites due to the overlapping by the intensity of a wide SO_4^{-2} band. The presence of aminoglycoside antibiotics in drug-loaded materials was indicated only by the appearance of the 1527 cm^{-1} band (N—H

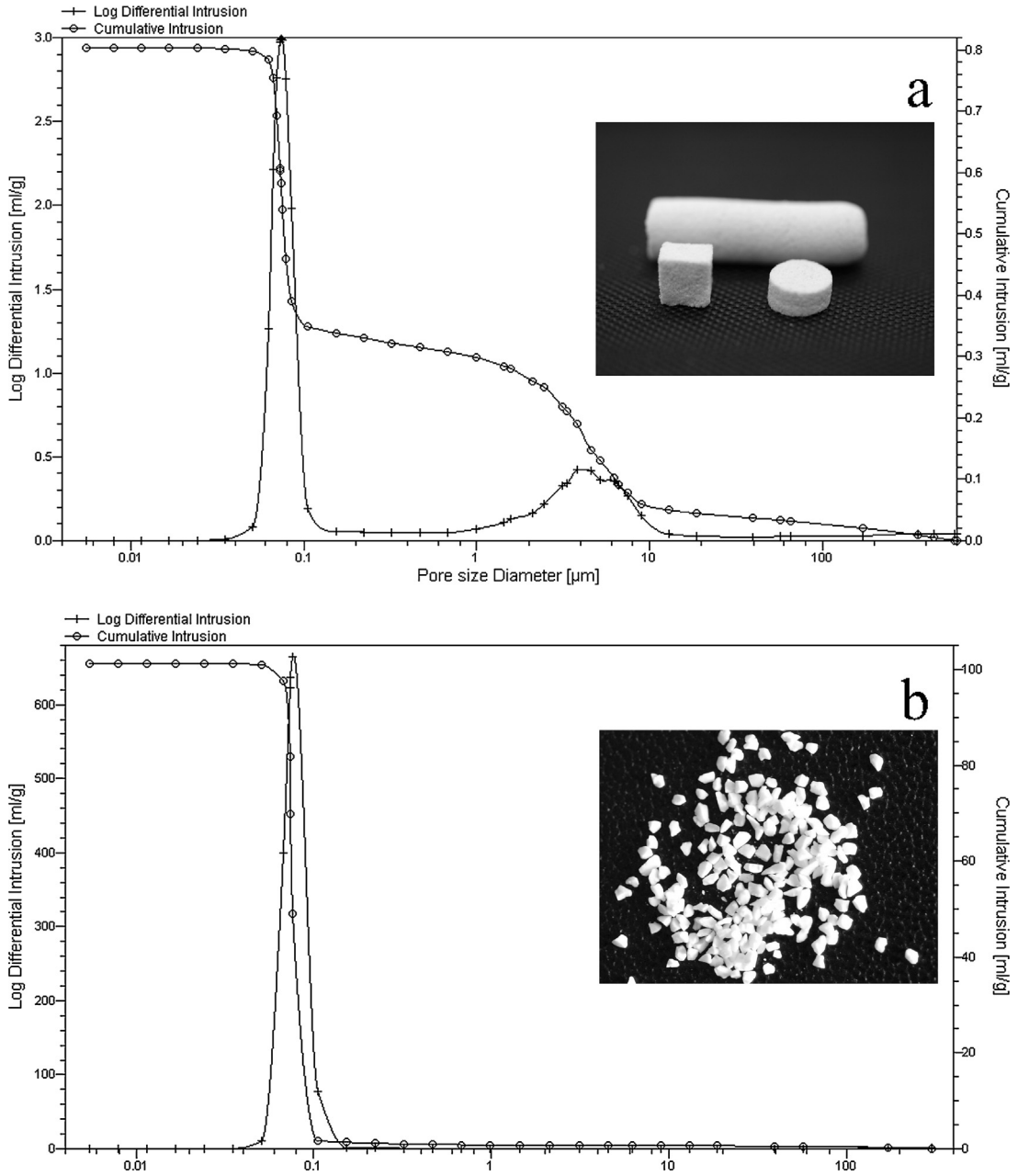


Fig. 2. Pore distribution in pure composite (a) and the highly porous HAp granules used for the composite preparation (b).

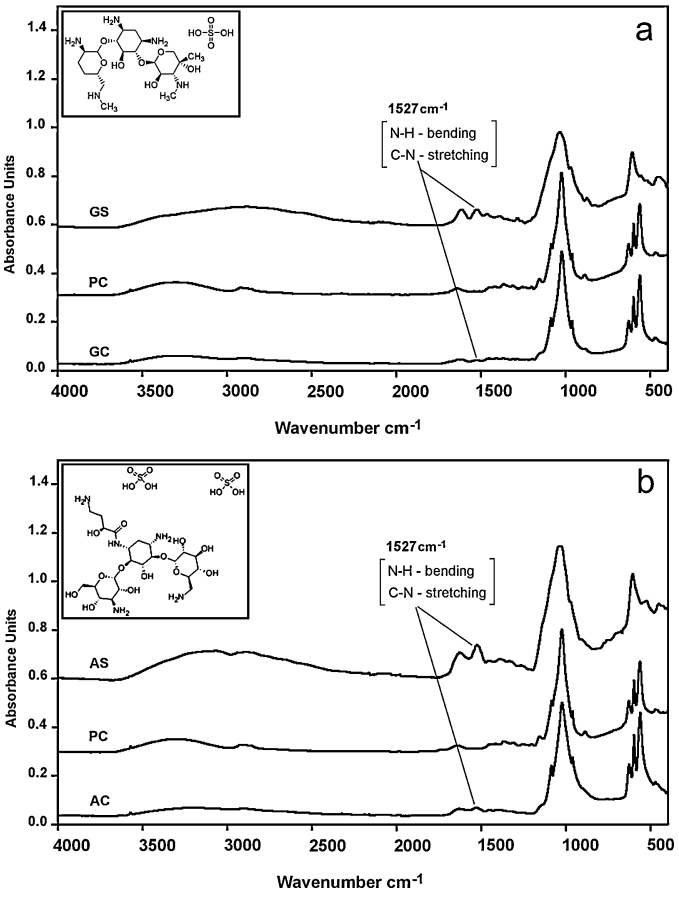


Fig. 3. FTIR spectra of gentamicin-loaded (a) and amikacin-loaded (b) composites. GS and AS, gentamicin sulfate and amikacin sulfate, respectively; PC, pure composite; GC and AC, gentamicin-loaded ($G_{1\%;M2}$) and amikacin-loaded ($A_{1\%;M2}$) composite, respectively. In insets, the structures of gentamicin sulfate and amikacin sulfate, respectively.

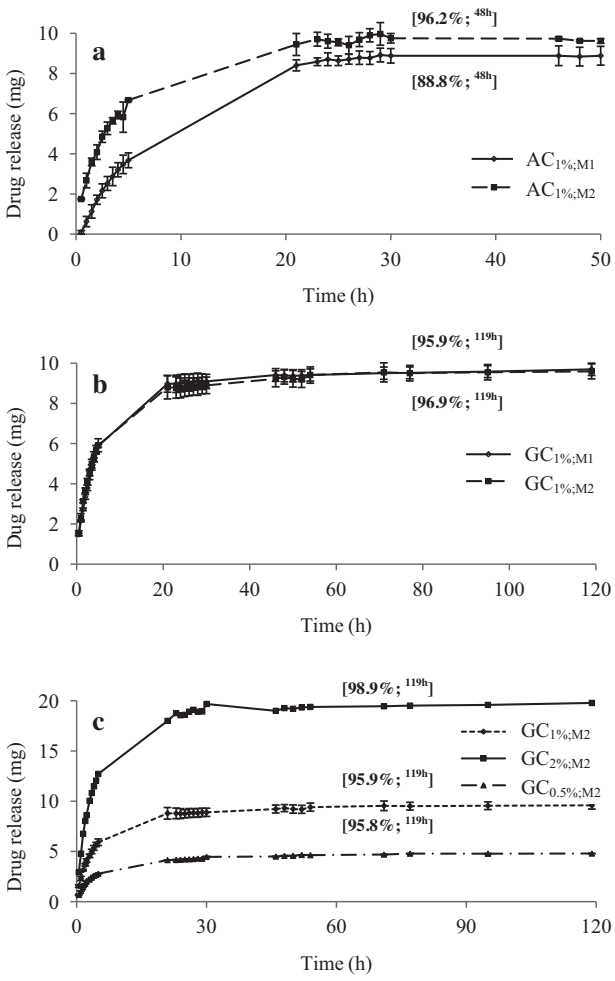


Fig. 4. Drug release profiles in the closed-loop system. 1% amikacin-loaded (methods M1 and M2) composite (a); 1% gentamicin-loaded (methods M1 and M2) composite (b); comparison of drug release profiles from the 0.5–2.0% gentamicin-loaded (method M2) composites (c). In square brackets: final values of the cumulative drug release; time to plateau (in superscript). AC, amikacin-loaded composite; GC, gentamicin-loaded composite.

bending and C—N stretching vibrations) as pure composite lacks amino groups in its structure. Although no other bands characteristics of the selected drugs were observed (due to the low antibiotic dosage in the composites), it seems from the spectra that neither gentamicin nor amikacin was involved in any chemical reaction with the HAp–glucan composite compounds. This supports the theoretical expectations because both the HAp granules and β-glucan lack the appropriate active groups capable of the formation of covalent bonds with aminoglycosides. These results, similarly to the data concerning the porosity of the material, suggested a pore-dependent nature of drug release.

3.2. Mechanism of drug release

To verify this hypothesis in practice, drug release profiles in a closed-loop system were obtained with composites loaded with antibiotics via 2 methods (M1 and M2). In the case of the amikacin-loaded composites, the majority of the drug was released during the first 24 h, reaching plateau in the 48th hour (Fig. 4a) of the experiment. However, only 89–96% of the drug was released from these composites, depending on the loading method (Fig. 4a), whereas in the case of the gentamicin-loaded samples, 96–97% of the loaded drug was released, mostly during the first 48 h, reaching plateau at the 119th hour (Fig. 4b). The remaining amount of amikacin was neither released nor residually attached to the composite, according to the test methods used (data not shown). For this reason, a comparison of the drug release profiles from the materials loaded with different doses of antibiotic was performed using only the gentamicin-loaded systems.

In most systems, the drug diffusion profile and coefficient are dependent on the drug concentration. However, in the case of the 0.5–2.0% gentamicin-loaded composites, the differences in this parameter were almost imperceptible, with the total drug amount (96–99%) released within 119 h (plateau phase) (Fig. 4c). The observation seems logical because of the very high gentamicin solubility in water and perfect sink conditions applied in the tested system which emulated the conditions in human body. The release profiles would probably differ if the doses of gentamicin loaded into the composites were much higher than 2%, however, the released drug concentration could be simultaneously lethal for the osteoblast cells.

According to the presented results, the aminoglycoside antibiotics were released from the elastic composites following the Fickian diffusion or anomalous transport model (Table 2). The Fickian diffusion is observed when drugs are loaded via method M2, by simply soaking the composite in an antibiotic solution. When the drug was loaded into the HAp granules (method M1), further used for composite preparation, the values of the release exponent were higher and reached 0.6 (Table 2). In all cases, the values of the release exponent suggested that pore-dependent diffusion was the governing factor that affected the drug transport.

Table 2
Mechanism of diffusional drug release from the composite samples.

Sample code (% M_0)	Equation[a]: $y = k \times t^n$			Drug release mechanism
	k	n	R	
$AC_{1\%;M1}$ (1%)	0.0941	0.6	0.98667438	Anomalous transport
$AC_{1\%;M2}$ (1%)	0.2265	0.5	0.99067195	Fickian diffusion
$GC_{1\%;M1}$ (1%)	0.2215	0.6	0.99898242	Anomalous transport
$GC_{1\%;M2}$ (1%)	0.2455	0.5	0.99848869	Fickian diffusion
$GC_{2\%;M2}$ (2%)	0.2560	0.5	0.99520906	Fickian diffusion
$GC_{0.5\%;M2}$ (0.5%)	0.2292	0.5	0.99561742	Fickian diffusion

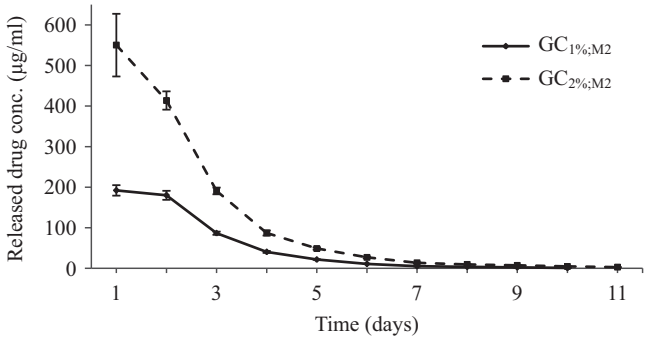
[a] Abbreviations for equation: % M_0, initial drug amount in dry composite (as a percentage of the initial composite weight); y, percent amount of the released drug in time (M_t/M_∞); k, kinetic constant; n, release exponent; t, time (h); R, correlation coefficient.

However, the influence of other mechanisms, such as the relaxation of β-glucan fibers during the composite soaking and the formation of weak ionic interactions between some antibiotic particles and the composite compounds, cannot be excluded. The effect of these mechanisms seems to be stronger in the case of a composite containing the drugs loaded into the HAp granules (method M1) than into the ready-to-use composite (method M2).

3.3. Antibacterial activity of drug-loaded composites

The estimation of the antibacterial activity of the gentamicin-loaded composite was performed in a semi-open system with a small rate of medium exchange, emulating in vivo conditions in the bone tissue where circulation of tissue liquids is very slow. The experiment included two stages (Fig. 1): stage one, concerning the estimation of the activity of the antibiotic released from the composites, and stage two, designed to evaluate the amount and the activity of the residual drug that remained after the elution of the removable amount. During the first stage, it was observed that – in the case of drug-free composite samples – all tested bacteria strains grew easily in the medium on the very first day of the experiment (Table 3), as it was expected due to the lack of a protective agent. Mueller–Hinton media incubated with 1% gentamicin-loaded samples were protected against the propagation of bacterial cells until the 9th day, with the exception of *P. aeruginosa* (8th day). The samples of the medium incubated with 2% gentamicin-loaded composites were free of bacteria until the 11th day, again with the exception of *P. aeruginosa* (10th day). These data were in accordance with the concentrations of the antibiotic released into PBS in an analogous experiment, as seen in the differential profiles (Fig. 5). In particular, in all cases the bacterial growth in the medium appeared when the concentrations of the released gentamicin decreased to the levels corresponding to the MIC values of gentamicin against particular bacterial strains (Table 3). Only in the case of *S. aureus*, the time-point of bacterial growth becoming visible was slightly surprising, as – based on the MIC values – it was expected to be comparable with that of *P. aeruginosa*. Sub-MIC concentrations of gentamicin were observed only in the last 1–2 days of the experiment (Fig. 5); this seems to be profitable for the prevention of the development of drug resistance. A significant similarity appeared between the periods of antibacterial protection of the culture medium observed in the case of the $GC_{1\%;M2}$ and $GC_{2\%;M2}$ composites (8–9 days and 10–11 days, respectively), although the loaded drug doses differed by 100%. The initial concentrations of the released drug were more than two times higher for $GC_{2\%,M2}$ than for $GC_{1\%;M2}$ (both below 1000 μg/ml, toxicity threshold for human osteoblasts). However, both profiles reached the values of 0.2–2.0 μg/ml (MICs of gentamicin against the tested strains) within the approximate time (Table 3). This suggests that the benefits resulting from the higher dose of antibiotic loaded into bone-substituting composites are not necessarily obvious and a lower dose of the drug may exhibit a sufficient efficacy.

The estimation of the activity of the drug remaining in the composite after the release of its soluble amount (Stage 2 of the experiment) proved helpful in explaining the complexity of the mechanism of its antibacterial action. In the presented experiments, the samples of the composite which were no longer capable of releasing the antibiotic were further incubated for 10 consecutive days in Mueller–Hinton medium containing a very high concentration of bacteria, normally not observed in human blood. Thus the experiment (including the estimation of both the released and the residual drug activity) lasted for a total of 11 days in the case of the control drug-free PC and for 20–21 days in the case of the drug-loaded $GC_{2\%;M2}$. The amount of bacteria adhered to the control composites was very high (10^6–10^8) on the first day of Stage 2 of the described experiment and remained at a similar level over the following 9 days (Table 4). The $GC_{2\%;M2}$ samples, although no longer able to inhibit the growth of the bacteria in the surrounding medium, were completely free of any adhered bacteria during the first 2–3 days of Stage 2 of the experiment. Subsequently, the samples became gradually colonized by the bacterial cells, but the rate of colonization was approximately 100 times lower than that of the control samples. An exceptional phenomenon was observed in the case of *E. coli* cells which started to adhere to the $GC_{2\%;M2}$ samples immediately and – on the 10th day of Stage 2 (21st day of the entire experiment) – colonized this biomaterial to a similar degree as with the PC samples (Table 4). The confocal microscopy technique allowed us to visualize the presence of numerous and mostly viable bacteria on the PC composite (Fig. 6a), in contrast to the $GC_{2\%;M2}$ samples on which much less bacteria, mainly dead and aggregated, were detected (Fig. 6b). The bacteria settled chiefly within the spaces between the ceramic granules (Fig. 6a). The results obtained



sample	Sub-inhibitory concentrations of released gentamicin (μg/ml) on day:						
	9	10	11	12	13	14	15
$GC_{1\%;M2}$	0.79	0.21	0	0	0	0	0
$GC_{2\%;M2}$	4.49	2.76	1.74	0.69	0.19	0	0

Fig. 5. Gentamicin release profile during Stage 1 of the estimation of antibacterial activity in the semi-open loop system (PBS as medium). In the table below: sub-inhibitory concentrations of the released drug in the last phase of the experiment. GC, gentamicin-loaded composite.

Table 3
Inhibition of bacterial growth in the culture medium by the drug released from the gentamicin-loaded composites in the semi-open loop system (MH broth as medium) during Stage 1 of the estimation of antibacterial activity. The gentamicin MIC values for the tested bacterial strains were: *S. aureus*: 2 μg/ml; *S. epidermidis*: 0.24 μg/ml; *E. coli*: 0.8 μg/ml; *P. aeruginosa*: 2 μg/ml.

Sample (day of Stage 1 of the experiment)	Bacterial strain	Occurrence of bacterial growth in the medium										
		1	2	3	4	5	6	7	8	9	10	11
PC	*S. aureus*	×										
	S. epidermidis	×										
	E. coli	×										
	P. aeruginosa	×										
$GC_{1\%;M2}$	*S. aureus*									×		
	S. epidermidis									×		
	E. coli									×		
	P. aeruginosa								×			
$GC_{2\%;M2}$	*S. aureus*											×
	S. epidermidis											×
	E. coli											×
	P. aeruginosa										×	

Table 4
Residual antibacterial activity and amount of residual drug in composite pieces during Stage 2 of the estimation of antibacterial activity (in the phase of the experiment following the occurrence of bacterial growth in the medium).

Bacterial strain	1st day	2nd day	3rd day	4th day	6th day	8th day	10th day
Amount of cells adhered to the control composite –PC (viable cells/1 g dry weight[a]). Medium, MH broth							
S. aureus	3.82×10^7	5.8×10^7	5.9×10^7	6.08×10^7	5.79×10^7	5.8×10^7	5.98×10^7
S. epidermidis	2.96×10^8	3.24×10^8	3.61×10^8	3.68×10^8	3.55×10^8	3.37×10^8	3.47×10^8
E. coli	1.09×10^8	1.71×10^8	1.79×10^8	1.88×10^8	1.91×10^8	1.74×10^8	1.79×10^8
P. aeruginosa	2.5×10^6	2.75×10^6	2.81×10^6	2.79×10^6	2.74×10^6	2.64×10^6	2.68×10^6
Amount of cells adhered to the 2%-gentamicin-loaded composite – $GC_{2\%;M2}$ (viable cells/1 g dry weight[b]). Medium, MH broth							
S. aureus	0	0	2.2×10^4	1.01×10^5	1.14×10^5	3.5×10^5	5.9×10^5
S. epidermidis	0	0	1.1×10^6	0.87×10^5	2.1×10^6	5.3×10^7	8.2×10^7
E. coli	2.3×10^4	4×10^5	2.1×10^5	4.1×10^4	1.71×10^7	1.61×10^8	8×10^8
P. aeruginosa	0	0	0	3×10^3	1.9×10^4	1.9×10^4	4.75×10^4
Elution medium	1st day	2nd day	3rd day	4th day	6th day	8th day	10th day
Amount of residual gentamicin in the 2%-gentamicin-loaded composite – $GC_{2\%;M2}$ (as % of the initial amount[SD]). Medium, PBS pH 7.4							
2 M NaCl	0.39 [3%]	0.38 [1.8%]	0.35 [6.2%]	0.37 [3.1%]	0.349 [3.6%]	0.364 [1.2%]	0.393 [5.4%]
H_2O	0.011 [5%]	0	0	0	0	0	0

[a]SD was in the range of 0.9–12%.
[b]SD was in the range of 0.7–8.1%.

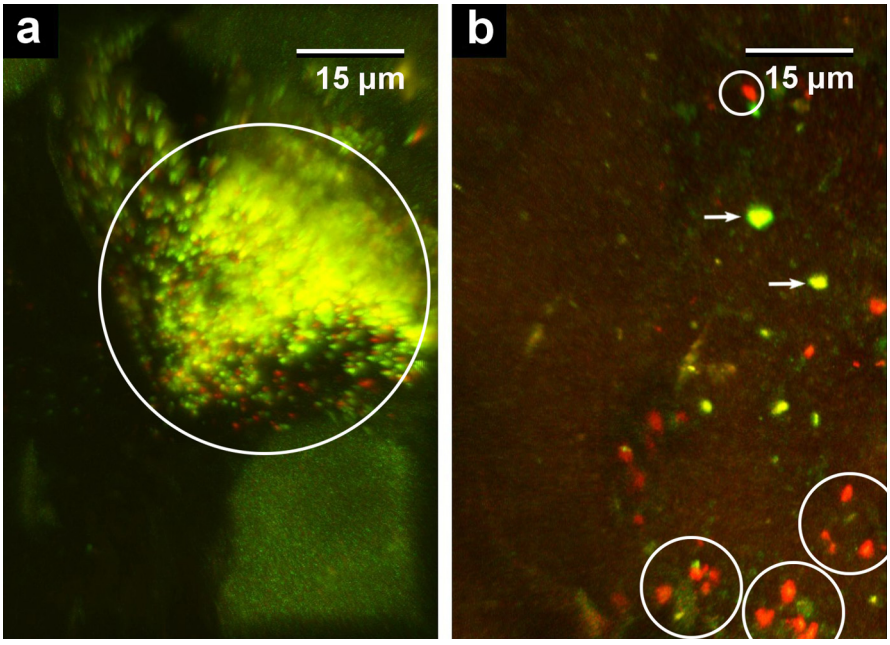


Fig. 6. Colonization of the control PC (a) and gentamicin-loaded $G_{2\%;M2}$ (b) composites by the *S. aureus* cells during Stage 2 of the estimation of antibacterial activity (within 10 days after the occurrence of bacterial growth in the MH culture medium). The arrows indicate the self-fluorescence of artifacts; the circles indicate the presence of bacteria. Viable and dead cells were stained green and red, respectively, and visualized using the confocal microscopy technique. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with the *S. aureus* strain, shown in Fig. 6, were very similar to those of the other tested bacterial strains.

The residual amount of gentamicin was eluted from the $GC_{2\%;M2}$ samples using a NaCl solution of high ionic strength (but not water). This amount constituted approximately 0.4% of the initial drug content and remained unchanged for 10 days of Stage 2 of the experiment, despite the continuous exchange of PBS (Table 4). This observation suggested that the residual amount of the antibiotic was bound to the composite via ionic interactions. The constant level of the residual amount of gentamicin in the material was not correlated with the level of protection against the bacterial adhesion because the latter gradually decreased during Stage 2 of the experiment. However, one should note that two different media were used in Stage 2 of the test: sterile PBS or Mueller–Hinton medium, with a very high content of live bacteria. Thus the influence of bacterial cells on the inactivation of the residual drug amount in the composites must be taken into account as a factor affecting the stability of the chemical interactions between the antibiotic molecules and the active groups on the composite surface.

4. Discussion

In most cases, the rate of infections of implantable materials is relatively low, due to a significant improvement in maintaining sterility in operating rooms and better perioperative care. Such infections occur in no more than approx. 4% of all cases. Therefore, the greatest risk of infection exists during the first period after a surgical procedure, before the restoration of soft tissue continuity, as a result of superficial wound infections spreading to the perioperative space or infected hematomas (type-I infections). However, type-II and type-III infections may develop after several months or years after the implantation as foreign bodies (implants) decrease the effectiveness of host defense mechanisms (Goel, 2006). Therefore, in the modeling of drug-loaded implantable materials, one must take into consideration both the short- and long-term antibacterial activity. The latter type is related with another problem concerning local antibiotic delivery – the induction of drug resistance in bacteria. All substances currently used in medicine may evoke this phenomenon, even silver ions, despite the common opinion that bacteria cannot develop resistance to this metal (Gupta et al., 1999; Silver et al., 2006; Zhao et al., 2011). Therefore, the optimal therapy in the case of standard implantation should include systemic antibiotic treatment combined with a relatively short-term local drug delivery from the biomaterial within the implantation site. Additionally, the period of drug release from the implant yielding sub-inhibitory concentrations should be as short as possible.

Taking into account all these factors, we prepared an elastic HAp–glucan composite loaded with aminoglycoside antibiotics for bone tissue regeneration. We paid particular attention to the following features of the composite: (i) it should release the majority of the drug into isotonic liquids relatively quickly; (ii) the concentration of the released drug should not exceed the toxicity threshold for human osteoblasts; (iii) sub-inhibitory concentrations of the released drug should not be observed; (iv) it should contain some quantity of residual drug, not eluted by liquids of ionic strength typical for bone tissue.

Aminoglycoside antibiotics-loaded HAp–glucan composites allowed the release of the majority of antibiotics within 48 h in a closed-loop system, with 89–99% of total drug eluted within up to 119 h (plateau phase). These results are similar to those obtained by Laurent et al. (2008) who reported a total gentamicin release from an HAp/TCP bone substitute in less than 48 h. This rate corresponds to the recommendations for antibiotic prophylaxis, in order to avoid the occurrence of drug-resistant bacterial strains. The initial concentration of the released gentamicin in the case of both drug-loaded composites did not exceed 600 μg/ml. As reported in our previous study (Belcarz et al., 2009b), the lethal gentamicin dose for hFOB (human fetal osteoblasts) amounts to 1000 μg/ml. Therefore, the concentration of the released drug detected in the presented experiments can be defined as not harmful for the tissues surrounding the implanted drug-loaded material. Thus the first two assumptions of our study were confirmed.

The cumulative drug release profile does not allow us to estimate the release of sub-inhibitory concentrations liberated just before the plateau phase, as such changes are very low in comparison with the final concentration of the released drug. For that reason, a semi-open release model was used to define the amount of antibiotic released at the end of the experiment. Sub-inhibitory concentrations of gentamicin in the PBS medium were observed for only 1–2 days. Simultaneously, it was found that approx. 0.4% of the initial drug content was attached to the compounds of the elastic composite via ionic bonds and remained within the biomaterial structure as a residual amount, not removable by water or the PBS solution. This demonstrates that the drug-loaded elastic composites confirm the other two assumptions of our study.

The mechanism of anomalous diffusion of drug release, observed in the case of some tested composites, refers to a combination of diffusion-dependent and polymer relaxation-dependent models of drug release. In our experiments, the values of the release exponents were equal or very close to 0.5, suggesting mainly pore-dependent nature of antibiotic release. However, the effect of the polymer presence seems to be beneficial due to its chemical structure. In particular, a small amount of the residual drug is attached to the composite via ionic interactions, probably between the amino groups of aminoglycoside antibiotics and the hydroxyl groups which are abundantly present in β-1,3-glucan. The same mechanism, based on the formation of ionic interactions between the gentamicin molecules and the negatively charged lipopolysaccharides of the bacterial outer membrane, is also responsible for the first stage of aminoglycosides uptake by bacteria (Becker and Cooper, 2013). We observed that, due to the presence of the drug both easily eluted and bonded by ionic interactions, the composites acted against bacteria in a biphasic mode. The easily eluted antibiotic inhibited the bacterial growth in the culture medium (the first phase). Subsequently, the residual and ionically bonded drug acted as a short-lived barrier against the attachment of bacterial cells to the composite, thus enhancing its local antibacterial protection and reducing the risk of a time-delayed infection (the second phase). These results are similar to those obtained in our previous study, concerning the mixed-type mode of gentamicin bonding to keratin-modified ceramic granules (Belcarz et al., 2009b). In the experiments described in this paper, ionic interactions occurred between the antibiotic molecules and keratin fibers.

The amount of the residual gentamicin, constituting approx. 0.4% of the initial drug content, remained unchanged in the composites during Stage 2 (10 days). However, the period of a complete resistance of the tested samples to bacterial adhesion during this stage of the experiment was relatively short (2–3 days). This inconsistency may be puzzling. However, it must be noted that the concentration of bacteria cells used in the described experiment was extremely high and corresponded to a massive local infection. Thus the bacteria may have acted as a factor influencing the stability of the composite–drug ionic bonds.

Providing an explanation to this phenomenon is difficult because the mechanism of action of aminoglycoside antibiotics against bacteria remains unclear. Aminoglycosides are concentration-dependent antibiotics and have an extended post-antibiotic effect. These drugs have been shown to accumulate in bacterial cells by means of active transport (Pegram, 2003; Becker

and Cooper, 2013). The exact mechanism of aminoglycoside uptake and accumulation is based on a quick initial reaction between the positively charged drug molecules and the negatively charged molecules of bacterial outer membrane (first stage), followed by the formation of transient holes and energy-dependent uptake of the antibiotic (second and third stage) (Becker and Cooper, 2013). The aforementioned negatively charged molecules include lipopolysaccharide (LPS) in Gram-negative bacteria and phospholipids or teichoic acids in Gram-positive strains. It has been found that the process of electrostatic binding of aminoglycosides to bacterial surface is sensitive to polycations which antagonize significantly the first stage of drug uptake (Taber et al., 1987). It is therefore possible that – in the case of the studied composite – the negatively charged bacterial compounds may compete with other polyanions (like glucans) for positively charged drug molecules. Thus, the gradual displacement of aminoglycosides from the composite may take place in direct contact with the bacterium. Subsequently, the released drug molecules may undergo ionic binding to LPS on bacterial outer membrane and initialize further steps of drug uptake.

Another possible explanation of the results observed in our experiments may lie in the instability of Mg^{2+} ion binding to bacterial envelope in the presence of other positively charged molecules. This mechanism has been explained for Gram-positive species. Mg^{2+} ions are normal counterions associated with the envelope of Gram-positive bacteria cells, contributing to its stability. Aminoglycosides (dihydrostreptomycin) have been found to readily displace Mg^{2+} from teichoic acids in vitro (Kusser et al., 1985). In our experiments, the molecules of gentamicin eluted from the composite might have displaced some Mg^{2+} ions from bacterial envelope. Subsequently, the displaced ions might have accumulated locally and – by inducing a local increase in ionic strength – disclosed the molecules of ionically bound gentamicin from the composite. Finally, the molecules of gentamicin released from the composite could undergo accumulation in bacterial cells.

According to these hypothetical models, it is possible that the gentamicin molecules, once accumulated within bacterial cells, remained "inactivated", which enabled other bacterial cells to gradually colonize the composite within its drug-free areas. This hypothesis is also in agreement with the microscopic observations of the $GC_{2\%;M2}$ composites during Stage 2 of the experiment. It was found that aggregated dead bacteria dominated over the live ones, in contrast to the control PC composites.

A question may arise, whether the addition of β-1,3-glucan to the composite is beneficial or harmful in the recapitulation of its properties. Laurent et al. (2008) emphasize that polymers, as nonosteoconductive materials, may negatively affect bone ingrowth by closing porosities or by covering the ceramic phase. However, the bimodal porosity of the composite, with polymer-dependent macropores (approx. 10 μm), may induce the ingrowth of bone cells and microvessels. The minimum pore size required for an effective bone ingrowth into the porous structures of calcium phosphate ceramics is reported to be approximately 100 μm (Hing et al., 1999). In the case of the tested elastic composite, the pores were no wider than 10 μm. However, the composite is partially based on a soft glucan polymer. Thus this phase is a hypothetical space for microvessels and bone ingrowth. Moreover, it is out of discussion that the β-glucan polymer is advantageous as it provides the composite with its elastic properties. The glucan also seems to be essential for the residual antibacterial activity, as it enables the antibiotic to ionically bond to the composite. This strategy, resembling that used in the formulation of rifampicin-loaded vascular prostheses sealed with succinylated gelatin (Strachan et al., 1991), resulted in a reduced bacterial adhesion to the composite. Although the level of thus obtained protection is lower than in the case of biomaterials containing covalently immobilized drugs (Pichavant et al., 2012; Belcarz et al., 2009b), the described strategy is characterized by a significant benefit: the lack of binding agents (e.g., glutaraldehyde, dicyclohexylcarbodiimide, etc.) which are usually toxic or allergenic.

Our presumptions on the creation of a good drug delivery system (DDS), listed above, are not in full agreement with other sources. The already mentioned Laurent et al. (2008) recommended a biomaterial which released a high dose of the drug within 48 h, without any residual drug content, for prophylactic treatment. On the other hand, Campoccia et al. (2010) described the perfect DDS as a material exhibiting a high rate of the initial drug release (to promptly eradicate the majority of bacteria) and a prolonged release (to release the drug for several weeks or months after the surgery in order to eradicate the delayed infections occurring more than 2 years later). However, Campoccia et al. also admitted that the systems designed for a prolonged drug release, with persistent sub-inhibitory concentrations of antibiotics, raise serious concerns. Such systems may possibly contribute to enhancing the biofilm formation and the selection of resistant strains. Numerous studies support their argumentation. Neut et al. (2003) reported a case in which the gentamicin-loaded PMMA balls induced the occurrence of drug resistant mutants 5 years after the implantation due to a local release of antibiotic at the level of 4 μg/ml. The chemical nature of the implant surface may be another factor affecting the emergence of antibiotic-resistant bacteria (Arciola et al., 2002). This clearly demonstrates the importance of the problem of bacterial resistance upon contact with implants of prolonged drug release. In light of these facts, our concept for drug-loaded elastic biomaterial, balancing between the two strategies mentioned above, seems to be promising for antibacterial prophylaxis, with minimized side effects.

5. Conclusion

We prepared a biphasic HAp–glucan composite of a very high porosity, loaded with aminoglycoside antibiotics, that presents elasticity and high surgical handiness. The composite demonstrates a biphasic mode of antibacterial action. In the first phase, it releases the majority of the drug during the first 48 h, with a very short period of drug release at sub-inhibitory concentrations. In the second phase, the composites bonded with antibiotics via ionic interactions (approx. 0.4% of the initial drug amount) provide an additional short-lived barrier against bacterial adhesion to the composite. This suggests that the composite can be recommended for antibiotic prophylaxis, in order to avoid the occurrence of drug-resistant bacterial strains. Its use is not advised in the case of revision operations aimed at removing the infected implant, in which the new implant is required to provide a very high and long-term antibacterial activity.

The appearance of ionic interactions between the composite and the drug suggests that other antibiotics, containing positively charged groups in their structure, may also be potentially used for the preparation of the drug-loaded elastic HAp–glucan composite, especially since simple soaking of the composite in the drug solution seems to be a sufficient method for its fabrication. Consequently, the surgeon could choose the appropriate antibiotic from the selected group for that particular patient. Therefore, further studies are required to select the antibiotics suitable for this purpose. Moreover, the research should focus on the estimation of the risk of occurrence of drug-resistant strains in the elastic HAp–glucan composite upon a long-term exposure.

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