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Amikacin-modified hybrid biomaterial antimicrobial properties

Właściwości przeciwbakteryjne hybrydowego biomateriału modyfikowanego amikacyną

Implant infections treatment is the most common problem at orthopaedics. The incidence of orthopaedic implant infections is often reported to be 1–4%. Orthopaedic implant infections treatment is expensive and long-lasting. Antibiotics are provided as prophylactics, either orally or intravenously. However, very little accessibility of the site of infections often prolongs the treatment [6]. Poor blood circulation in osseous tissue is the main cause of the reduced therapeutic effects of administered drugs. On the other side increasing doses of antibiotics can cause side-effects [7, 8]. Researchers try to design biomaterials which protect their surface from bacterial colonization using antimicrobial agents, because biofilm formation on implant surface requires reoperation, implant removal and causes the patient's suffering.

Aminoglycoside antibiotics (amikacin or gentamicin) immobilization on hydroxyapatite ceramics (HAp) could be very profitable to fight this problem. Amikacin shows biological activity against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa* (strains responsible for the majority of infections at orthopaedics and maxillofacial surgery departments).

Forming a new kind of hybrid biomaterial with antimicrobial activity was the main aim of this research.

MATERIAL AND METHODS

BIOMATERIALS

Hydroxyapatite (HAp) was made in the Department of Technology of Ceramics and Refractories, AGH-University of Science and Technology, Cracow, Poland. Hydroxyapatite parameters were: diameter: 0.3–0.5 mm, open porosity: 67%, sintering temperature: 800°C.

IMMOBILIZATION PROCESS

A portion of HAp was covered with γ-aminopropyltriethoxysilane and divided into three parts. Two parts of silanized-HAp were chemically modified by two kinds of protein (porcine gelatin or keratin derived from human hair) according to Weetall [13] procedure in authors' own modification. Therefore, three types of matrix were obtained (silanized-HAp, gelatin-HAp and keratin-HAp). Amikacin (Biodacyna®, Bioton, Poland; 250 mg/ml) was immobilized according to the Polish Patent [4] and its concentration was estimated spectrophotometrically after Ginalska et al. [5].

IN VITRO DRUG RELEASE

Portions of HAp (0.5 g) were placed in tubes containing 5 ml of sterilized PBS (pH 7.4) and incubated at 37°C. Every 24 hours, 1 ml (20%) of total volume was taken and replaced by fresh PBS. Amikacin concentration was estimated in the samples. Antibiotic concentration was measured until its decreased below the detection minimum.

BACTERIAL STRAINS AND MEDIA FOR BACTERIAL GROWTH

Immobilized antibiotic biological activity was tested in the presence of *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* methicillin-resistant MR3 – strain from National Reference Center for Antibiotic Resistance and Surveillance, National Medicines Institute, Poland; *Staphylococcus epidermidis* ATCC 12228 and *Pseudomonas aeruginosa* ATCC 27853. Mueller-Hinton broth and Mueller-Hinton agar (Oxoid, England) were used in microbiological tests.

MICROBIOLOGICAL TESTS

a) Antimicrobial activity

S o l i d m e d i u m t e s t . Solid media in Petri dishes were inoculated with a bacterial suspension. Next, 50 mg, portions of different kinds of HAp were placed on inoculated agar medium. Petri plates were placed at 37°C for 18 hours. After that, time bacterial inhibition zones were measured.

B r o t h m e d i u m t e s t . 0.5 g portions of modified-HAp (HAp-silan-amikacin, HAp-gelatin-amikacin, HAp-keratin-amikacin) and non-modified HAp (only soaked in amikacin solution) were placed in closed tubes containing 5 ml of Mueller-Hinton broth. Next, 15 µl of bacterial suspension (0.5 McFarland) was added. After 24 hours, 1 ml (20%) of total volume was taken and replaced by fresh Mueller-Hinton broth and inoculated with 15 µl fresh bacterial suspension again. When the growth of bacteria in broth was observed, the experiment was finished.

b) Bacteria adhesion test

The next test purpose was to check if bacteria adhere to HAp surface. This test was made by incubating HAp granules (from the above-mentioned experiment) with 0.001% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma USA) in Mueller-Hinton broth for 24 h at 37°C [10]. The appearence of a purplish red formazane on HAp surface confirmed a living bacteria presence.

RESULTS

AMIKACIN IMMOBILIZATION

In our previous research, it was found that the drug was attached to the modified matrices in a mixed way: by physical adsorption (removable with water), by ionic interactions (removable with NaCl) and via covalent bonds (non-removable using the above solvents) [14]. In this study we used modified HAp granules which were not washed with water nor NaCl (HAp with all types of chemical interactions). Amounts of immobilized amikacin are presented in: Table 1 description, Table 2 and Table 3. It was found out that during chemical immobilization a double amount of amikacin was attached to HAp in contrast to soaking procedure (Table 1 description, Table 2 and Table 3).

Table 1. Bacterial strains inhibition growth zones on solid medium (Mueller-Hinton agar) in the presence of various types of HAp

Bacterial strains	*S.aureus* ATCC 25923 growth inhibition zones (mm)			
	HAp only soaked in amikacin[1]	HAp-amikacin[2]	HAp-gelatin-amikacin[3]	HAp-keratin-amikacin[4]
S. aureus	25	33	34	34
S. aureus MR3	25	33	32	33
S. epidermidis	28	34	34	36
P. aeruginosa	25	32	32	33

Amounts of antibiotic on 50 mg portions of HAp granules. [1]HAp soaked in amikacin – 0.24mg; [2]HAp-amikacin – 0.40mg; [3]HAp-gelatin-amikacin – 0.38mg; [4]HAp-keratin-amikacin – 0.41mg

Table 2. HAp granules antimicrobial activity against bacterial strains in time

Type of HAp	Amount of antibiotic on HAp (mg/g)	Day of broth contamination			
		S. aureus ATCC 25923	S. aureus MR3	*S. epidermidis* ATCC 12228	*P. aeruginosa* ATCC 27853
Non modified-HAp (soaked in amikacin)	4.88	24	18	18	19
HAp-amikacin	8.12	21	13	22	16
HAp-gelatin-amikacin	7.60	26	20	25	21
HAp-keratin-amikacin	8.20	28	23	23	23

Table 3. Influence of sterilization type on HAp-protein-amikacin granules antimicrobial properties (*S. aureus* ATCC 25923)

HAP granules type	Amount of antibiotic on HAp (mg/g)	Day of broth contamination		
		UV rays sterilization	Ethylene oxide sterilization	Steam sterilization
HAp-gelatin-amikacin	9.92	30	31	24
HAp-keratin-amikacin	9.56	33	35	27

AMIKACIN RELEASE

It was shown that modified carriers (HAp-amikacin, HAp-gelatin-amikacin, HAp-keratin-amikacin) released antibiotics at a similar rate. HAp granules soaked only in antibiotic solution released a greater amount of amikacin than modified carriers during the first fifteen days of our study. After 24 hours from experiment begining amikacin concentration released from HAp-control was about 180 μg/ml and 120 μg/ml for modified carriers. Higher drug concentrations released from HAp-control were probably caused by the fact that antibiotics were immobilized on HAp-control granules by adsorption and ionically and their elution was easier (Figure 1).

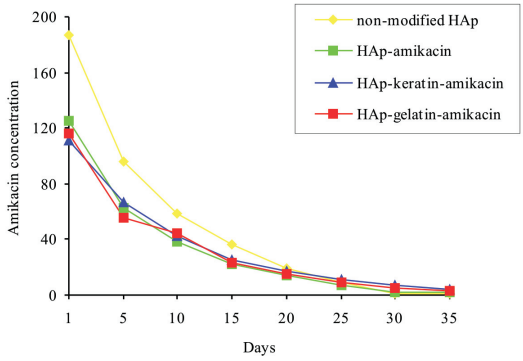

Fig. 1. Amikacin elution profile from HAp granules. Amounts of amikacin attached to 0.5g HAp: HAp-control – 2.44 mg; HAp-amikacin – 4.06 mg; HAp-gelatin-amikacin – 3.80 mg; HAp-keratin-amikacin – 4.10 mg

HAP GRANULES ANTIMICROBIAL ACTIVITY

Modified-HAp (HAp-amikacin, HAp-gelatin-amikacin or HAp-keratin-amikacin) and non-modified HAp used as control (HAp-amikacin) were examined in our study. Tests on solid medium (Table 1) showed that all types of HAp inhibited bacterial growth. However, HAp-amikacin, HAp-gelatin-amikacin and HAp-keratin-amikacin zones had greater sizes (32–36 mm) than HAp-control zones (25–28 mm). Data presented in Table 2 lead to the conclusion that all types of HAp granules inhibited bacteria's growth in broth medium and showed prolonged antimicrobial activity. The most important fact was that chemically immobilized amikacin on protein-HAp surface protected biomaterials from bacterial adhesion (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* MR3, *Staphylococcus epidermidis* ATCC 12228 and *Pseudomonas aeruginosa* ATCC 27853) and biofilm formation (Figure 2). In contrast, living bacteria were observed on HAp soaked in amikacin solution (HAp-control) and HAp-amikacin surfaces (purplish red colour formazane). Therefore, covering HAp surface by protein and amikacin immobilization on this surface was very advantageous, because protected HAp from biofilm formation.

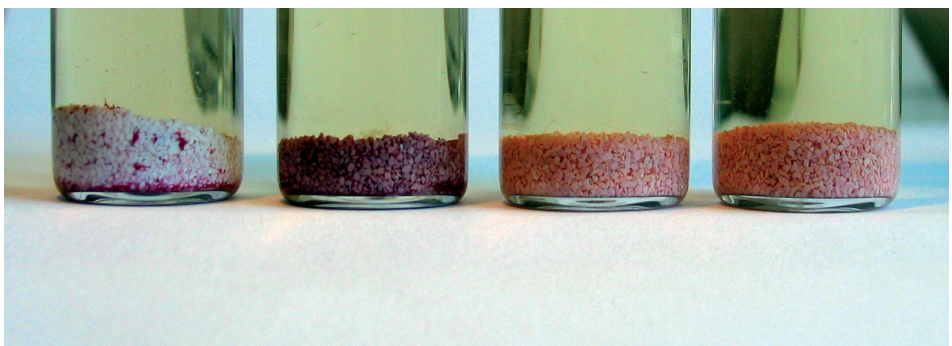

Fig. 2. Bacterial adhesion (*S. aureus)* to HAp surface: a – HAp soaked in amikacin, b – HAp-silan-amikacin, c – HAp-silan-gelatin-amikacin d – HAp-silan-keratin-amikacin

STERILIZATION PROCESS INFLUENCE ON HAP GRANULES ANTIMICROBIAL ACTIVITY

Biomaterials before implantation have to be sterilized and they should be resistant to sterilization process. The next experiment concerned/verified sterilization influence on antibiotic activity. It was shown that amikacin attached to HAp surface was still active towards *Staphylococcus aureus* ATCC 25923 strain (Table 3). Sterilized granules inhibited bacterial growth for the same time as non-sterilized granules. It results from this that sterilization process did not damage immobilized amikacin structure.

HAP GRANULES STORAGE TIME INFLUENCE ON THEIR ANTIBACTERIAL ACTIVITY

The last step of this research concerned checking the influence of time on HAp granules storage and testing their antimicrobial activity again. It was found out that HAp granules could be stored even two years at room temperature without losing their biological activity. After the experiment lasting three weeks (procedure description in *Microbiological tests*) there was no bacteria growth in broth medium.

DISCUSSION

A systemic antibiotic therapy after implantation can cause some problems. It is dificult to achieve an adequate drug concentration at the infection site (high antibiotic concentration in serum is toxic) and there is a risk of implant surface bacterial colonization and biofilm formation. Biofilm protects bacteria from antibiotics and host immunological response [2] The requirement to search for new biomaterials with antimicrobial activity is widespread in the biomaterials engineering field. Researchers try to design biomaterials which protect their surface from bacterial colonization using antimicrobial agents. Applications of antibiotic bone cements, Septopal® (PMMA chains and minichains loaded with gentamicin sulphate), collagen sponge with gentamicin, irrigation drainage or ozonotherapy are the routine methods of implant infections prevention, but they are not sufficient [3].

Polymethylmethacrylate (PMMA) is used in orthopaedic surgery to fix prosthetic components and as an antibiotic carrier. Its porous structure enables slow release of antibiotic and it is possible to achieve high antibiotic concentration *in situ*. Unfortunatelly, PMMA polymerization process is egzothermic (temperature raises above 80°C) and causes bone tissue local necrosis [9]. Also, the antibiotic which is added to PMMA has to be resistant to PMMA polymerization temperature. This limited the application of many antibiotics. In contrast, HAp ceramics could be loaded with many antibiotics [12]. Our study showed that antibiotics immobilization process did not influence negatively their biological properties. Septopal® is used as an antibiotic drug carrier, but it has to be removed to avoid toxic effect of PMMA and next an operation is required. Bacteria which have adhesion ability regardless of their antibiotic sensitivity could also colonize PMMA chains surface. In comparison, HAp ceramics, which is widely used in orthopaedics as a bone substitute, do not show toxic effects on patient's tissues. Its main advantages are biocompatibility, osteoconductivity and bioactivity. These features are confirmed in some studies [1, 11]. In our research, both non-modified HAp (only soaked in amikacin) and modified HAp protected broth from bacterial growth for about 1 month, but after this time we could observe living bacteria on non-modified HAp and HAp-amikacin surface in contrast to modified granules (HAp-protein-amikacin) where living bacteria were not present. Our studies proved that antibiotic chemical immobilization on protein (gelatin or keratin) covered HAp protected its surface from biofilm formation. The time of biomaterial protection depended on the amount of

immobilized amikacin and the kind of HAp modification. Immobilization method did not influence amikacin antimicrobial properties. We could observe bacterial growth inhibition by modified-HAp. The antibiotic attached to biomaterial *via* weak chemical interactions (adsorption) was released to the surrounding environment at the beginning of the experiment. It ensured early protection against bacteria. Next, amikacin connected with HAp granules by ionic bonds, amikacin present in HAp granules pores and antibiotic entrapped among protein fibres used to HAp modification was released. Covalently bound antibiotic showed protection in the last phase.

Sterilization processes (UV radiation, ethylene dioxide, steam sterilization in an autoclave) also did not damage amikacin structure and did not remarkably influence the protection time. Protection shorter time of broth medium was only observed in case of autoclaving. It could be caused by the temperature of this process (121°C).

Stability of our biomaterial hybrid is the next important advantage. After two years of storage biomaterial showed similar antimicrobial activity to the activity obtained soon after amikacin immobilization process.

CONCLUSIONS

Antibiotic-modified HAp has antimicrobial activity against *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeuruginosa* (strains responsible for the majority of infections at orthopaedics and maxillofacial surgery departaments) and is resistant to biofilm formation. Antibiotics are bound strongly, their elution from HAp is slow and the desired amount of the drug could be immobilized on HAp surface. Moreover, sterilization process and storage time do not influence biomaterial antimicrobial properties.

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SUMMARY

Orthopaedic implant infections are a serious problem in present-day medicine. This study is a trial of a new type biomaterial with antimicrobial properties formed by chemical antibiotic immobilization on HAp granules surface. Biomaterial was protected from bacterial adhesion (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* MR3, *Staphylococcus epidermidis* ATCC 12228*, Pseudomonas aeruginosa* ATCC 27853) and biofilm formation. Biomaterial hybrid showed prolonged antimicrobial activity (20–30 days). Biomaterial was resistant to sterilization process and saved its biological activity. Amikacin-modified HAp granules with regard to its properties could be a profitable biomaterial for future medical applications.

STRESZCZENIE

Zakażenia implantów ortopedycznych stanowią istotny problem we współczesnej medycynie. Badania przedstawione w pracy są próbą opracowania nowego typu biomateriału, posiadającego właściwości przeciwbakteryjne dzięki unieruchomieniu antybiotyku na powierzchni granulatu HAp. Utworzony biomateriał był chroniony przed adhezją bakterii (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* MR3, *Staphylococcus epidermidis* ATCC 12228 i *Pseudomonas aeruginosa* ATCC 27853) i wytworzeniem przez nie biofilmu. Hybrydowy biomateriał wykazywał przedłużoną aktywność przeciwbakteryjną. Ponadto był on odporny na proces sterylizacji i zachował swoje biologiczne funkcje podczas przechowywania w temperaturze pokojowej. Zmodyfikowany lekiem granulat hydroksyapatytowy ze względu na swoje właściwości wydaje się korzystnym biomateriałem dla przyszłych zastosowań medycznych.