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Two-component biocomposite: chitosan – hydroxyapatite. Preparation and estimation of properties

Dwuskładnikowy biokompozyt: chitosan – hydroksyapatyt. Opracowanie i ocena właściwości

INTRODUCTION

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (HAp) is an interesting biomaterial with potential orthopedic, dental, and maxillofacial applications due to its excellent biocompatibility, bioactivity, and osteoconductivity [4]. HAp, being chemically and structurally similar to the inorganic component of bone, enamel, and dentin, has received considerable attention from the biologists and biomaterial scientists. It has been successfully used as bone fillers, coating of orthopedic implants and cell-culture carriers. Another property is that HAp can be combined with a variety of biomaterials such as alginate, hyaluronic acid, calcium phosphate, poly-L-lactic acid and chitosan. In our research we focused on hydroxyapatite-chitosan composite with specific properties appropriate for clinical requirements. Chitosan is a product obtained from the de-N-acetylation of chitin, a co-polymer of N-acetyl-glucosamine and N-glucosamine units linked by β-(1→4) glycosidic bonds, in the presence of hot alkali. Chitosan also has intrinsic antimicrobial activity against bacteria and fungi [9, 12, 5]. To intensify these antimicrobial properties, researchers added antibiotics to composites to be used for orthopedic surgery. Using antibiotic delivery system is very important to prevent implant infections [13]. Gentamicin immobilization on HAp-chitosan biomaterials can significantly increase antimicrobial properties of this biomaterial.

In the present work, we aim to make a new hybrid composite with antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*.

MATERIALS AND METHODS

B i o m a t e r i a l s. Hydroxyapatite (HAp) was developed at 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: 800ºC.

Chitosan was obtained from krill chitin in Department of Food and Environment Chemistry, National Marine Fisheries Research Institute, Gdynia, Poland. Deacylation degree was 91.6% and molecular weight was 218 kDa.

I m m o b i l i z a t i o n p r o c e s s. A portion of HAp was covered by γ-aminopropyl-triethoxysilane. Silanized-HAp was chemically modified by chitosan according to Weetall's [15] procedure with authors' own modification. Gentamicin (Krka, Slovenia; 40 mg/ml) was immobilized according to the Polish Patent [8] and its concentration was estimated spectrophotometrically after Frutos-Cabanillos [3].

I n v i t r o d r u g r e l e a s e. Portions of HAp (0.5 g) were placed in tubes containing 5 ml of sterilized PBS (pH 7.4) and incubated in 37°C. Every 24 hours 1 ml (20%) of total volume was taken and replaced by fresh PBS. Gentamicin concentration was estimated in these samples. Antibiotic was measured until its concentration decreased below the detection minimum.

H y d r o x y a p a t i t e – c h i t o s a n m o u l d e r f o r m a t i o n. Two types of matrix were made. Chitosan (50 mg) was mixed with 1 ml 0.5% acetic acid and 70 mg HAp was added. Next, the first type of moulder was formed. At second experiment 50 mg of chitosan was mixed with 0.5 ml 0.5% acetic acid and 0.5 ml 20% gelatin solution. After precise components mixing, 70 mg HAp was added and the second matrix type was made. Moulders were placed in thermostat at 40°C for 1 hour. After that time moulders were placed in tubes with 5ml of PBS. Molders texture was observed during 72 h.

B a c t e r i a l s t r a i n s a n d m e d i a f o r b a c t e r i a l g r o w t h. Immobilized antibiotic biological activity was tested in the presence of Staphylococcus aureus ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Escherichia coli* ATCC 25992.

Mueller-Hinton broth and Mueller-Hinton agar (Oxoid, England) were used in microbiological tests.

M i c r o b i o l o g i c a l t e s t s

a) A n t i m i c r o b i a l a c t i v i t y

S o l i d m e d i u m t e s t. Solid medium in Petri dishes was inoculated with bacteria suspension. Next, 50 mg portions of HAp were placed on inoculated agar medium. Petri plates were placed in 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. Portions (0.5 g) of modified-HAp (HAp-chitosan-gentamicin), non-modified HAp (only soaked in amikacin solution) and HAp (control) 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 1 ml fresh Mueller-Hinton broth and inoculated with 15 µl fresh bacterial suspension (0.5 McFarland) again. When the growth of bacteria in broth was observed, the experiment was finished.

b) B a c t e r i a a d h e s i o n t e s t

The next purpose of the test was to check if bacteria adhere to HAp surface. This test was made by incubation of HAp granules (from above-mentioned experiment) with 0.001% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma USA) in Mueller-Hinton broth for 24 h at 37°C (7). The appearence of a purplish red formazane on HAp surface confirmed a living bacteria presence.

RESULTS

G e n t a m i c i n i m m o b i l i z a t i o n. In our previous studies we showed that antibiotic was attached to the modified matrices in mixed way: by physical adsorption (removable with water), by ionic interactions (removable with NaCl) and via covalent bonds (non removable using above solvents) [7,8,17]. In this research we used modified HAp-chitosan 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, Table 2. It turned out that during chemical immobilization almost triple amount of gentamicin was attached to Hap-chitosan in contrast to soaking procedure (Table 1, Table 2).

Non-modified carrier (control) had the ability to bind gentamicin by physical adsorption (35%) and by ionic interaction (24%); however, 61% was entrapped deeply in HAP pores. To chitosan-modified carrier about 52% of drug was bound by physical adsorption, 13% by ionic interactions, whereas the remaining amount of 35% antibiotic was partially attached via covalent bonds and partially entrapped within the pores.

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

Bacterial strains	Growth inhibition zones (mm)		
	HAp (control)[1]	HAp only soaked in gentamicin[2]	HAp-chitosan-gentamicin[3]
E.coli	0	28	25
S. aureus	0	30	30
S. epidermidis	0	37	34

Amounts of antibiotic on 50 mg portions of HAp granules. [1]HAp (control) – 0.00mg; [2]HAp soaked in gentamicin – 0.057mg; [3]HAp-chitosan-gentamicin – 0.163mg

Table 2. Antimicrobial activity of HAp granules against bacterial strains in time

Type of HAp	Amount of antibiotic on HAp (mg/g)	Day of broth contamination		
		E.coli ATCC 25992	*S. aureus* ATCC 25923	*S. epidermidis* ATCC 12228
HAp (control)	0.00	1	1	1
Non modified-HAp (soaked in gentamicin)	1.14	38	24	33
HAp-chitosan-gentamicin	3.25	40	25	35

Gentamicin release. It was found that granules soaked only in antibiotic solution released more amount of gentamicin than HAp-chitosan-gentamicin carrier during first five days of our study. After 24 hours from the beginning of the experiment gentamicin concentration released from non modified-HAp was about 247 µg/ml and 120 µg/ml for modified carrier. Higher drug concentrations released from HAp soaked in drug were probably caused by the fact that antibiotic were immobilized on HAp granules by adsorption and ionicly and their elution was easier. After that time both carriers released antibiotics in similar rate. Gentamicin release from HAp-chitosan-gentamicin biocomposite is slower, because a part of antibiotic was placed between chitin chains and it made drug elution difficult (Figure 2).

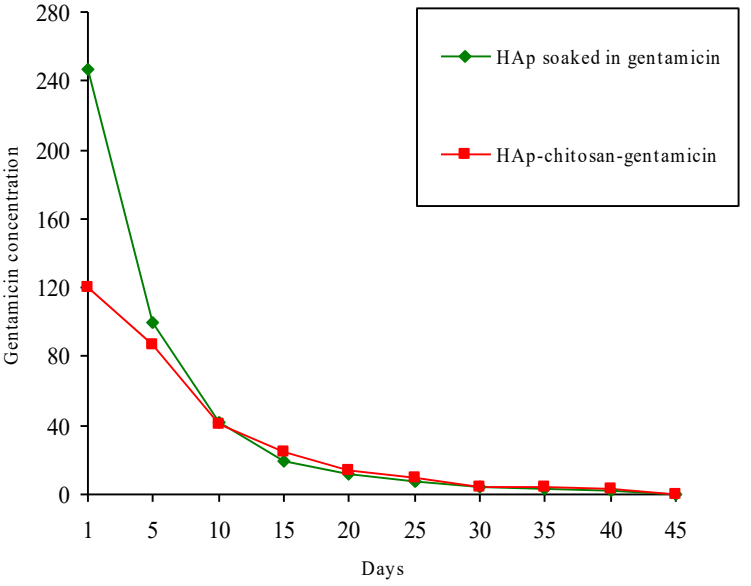

Figure 1. Gentamicin elution profile from HAp granules. Amounts of antibiotic attached to 0.5g HAp: HAp soaked in drug – 0.570 mg; HAp-chitosan-gentamicin – 1.625 mg

HAp granules antimicrobial activity. Two types of antibiotic carrier were examined in our study. Modified-HAp (HAp-chitosan-gentamicin) and HAp soaked in antibiotic; HAp without drug was used as control. Tests on solid medium (Table 1) showed 2 types of HAp with drug inhibited bacterial growth in opposition to HAp-control. Data presented in Table 2 leads to the conclusion that these

HAp granules also inhibited the growth of bacteria in broth medium and showed prolonged antimicrobial activity. The most important fact was that chemically immobilized gentamicin on HAp-chitosan surface protected biomaterial from bacterial adhesion (*Escherichia coli* ATCC 25992, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228) and biofilm formation (Table 3). In contrast, living bacteria were observed on HAp soaked in gentamicin solution (HAp-control) surface (purplish red colour formazane).

Table 3. Bacterial adhesion (S.epidermidis) estimation to HAp surface:
(-) – no adhesion, (+) – biomaterial bacterial adhesion

Carrier type	TTC reduction test after incubation in 37°C		
	1 h	4 h	24 h
HAp control	-	-	+
HAp soaked in antibiotic	-	-	+
HAp-chitosan-gentamicin	-	-	-

The influence of sterilization process on HAp granules antimicrobial activity. Biomaterials before implantation have to be sterilized and their properties should not be changed during sterilization process. It is particularly important when the implant is a drug carrier. Inappropriate sterilization process can damage the drug structure and inhibit its therapeutic activity. Next experiment verified sterilization influence on antibiotic activity. It turned out that gentamicin attached to HAp surface was still active towards *Staphylococcus aureus* ATCC 25923 strain (Figure 2). This study confirms that sterilization process did not damage immobilized antibiotic structure.

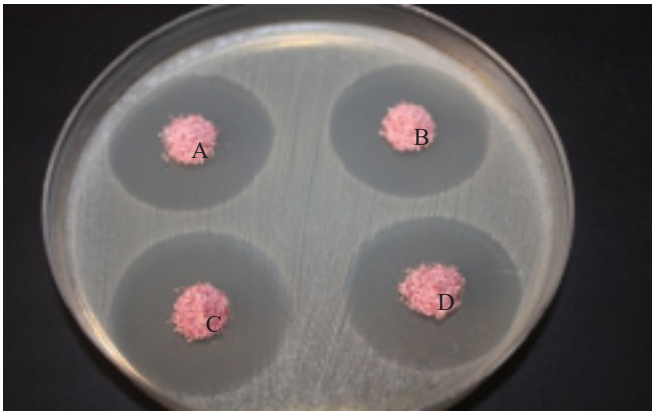

Figure 2. Sterilization type influence on HAp-chitosan-gentamicin granules antimicrobial properties (S. aureus ATCC 25923) a – non-sterilized HAp, b – UV sterilization, c – ethylene oxide sterilization, d – steam sterilization

Hydroxyapatite–chitosan moulder assessment. The last step of this study focused on checking the influence of PBS on HAp moulders structure. HAp mixed with chitosan or chitosan and gelatin, becomes a plastic mass which can be easily formed into various shapes (Figure 3).

It was showed that HAp granules mixed with chitosan did not change its shape during 72 hours incubation in PBS at 37°C. The moulder made from HAp, chitosan and gelatin started to to fall apart after the first hour of experiment. (Fig. 4).

Figure 3. Various shapes of moulders

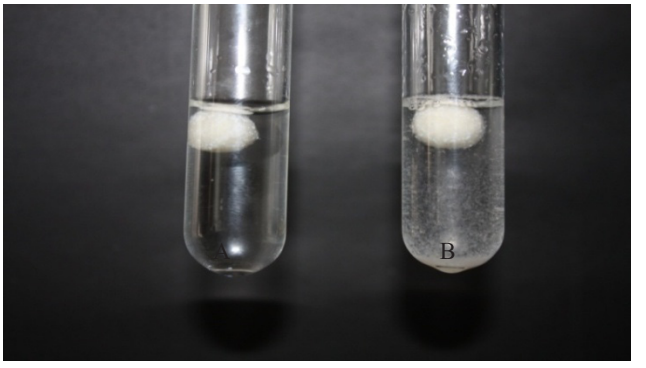

Figure 4. Moulders incubated in PBS at 37°C: a – HAp-chitosan; b – HAp-chitosan-gelatin

DISSCUSION

Every orthopedic surgery involves the risk of wound or implant contamination. When maintaining steady therapeutic drug concentration levels in living organisms it is a major issue to avoid potential contamination. When using oral drug administration, the potential disadvantages of such drug therapy include: high plasma concentrations of drugs that may lead to toxicity or low antibiotic levels caused at subtherapeutic blood levels, and, potentially, cause drug resistance in some instances [1]. That is why researchers test implants which have drug delivery systems. Controlled drug delivery systems can deliver precise dose of therapeutic agents to the target site. Generally, the controlled drug delivery

systems can maintain the concentration of drugs in the precise sites of the body within risk of overdosing [14] Drug delivery systems avoid contamination and biofilm formation on implant surface.

Numerous bacterial species have been described to form biofilms. Most commonly in orthopaedic surgery, Staphylococcus aureus, S. epidermidis, group A Strep, and Pseudomonas aeruginosa are the underlying causative organisms of musculoskeletal infections. All of these have been found to exist in a biofilm configuration. [11] Hydroxyapatite is ofen used as a drug delivery system, it can be mixed with other substances e.g. alginate, hyaluronic acid chitosan. In our study we concentrated on biocomposite made of HAp and chitosan. A very interesting property of chitosan is that it can be moulded in various forms such as powder, paste, film, fiber, and so forth, for application with different demands [12].

In our study both non-modified HAp (only soaked in antibiotic) and modified HAp (HAp-chitosan-gentamicin) protected broth from bacterial growth for 24 to 40 days depending on bacterial strain, but after this time we observed living bacteria on HAp-control and HAp soaked in antibiotic surfaces. In contrast to modified granules (HAp-chitosan-gentamicin) where living bacteria were not present. Our experiments showed that antibiotic chemical immobilization on chitosan covered Hap, protected its surface against biofilm formation. This observation is analogous to our previous experiments concerning drug immobilization on HAp-protein granules (HAp gelatin or HAp-keratin) [18]. The time of biomaterial protection depended on the amount of immobilized antibiotic. In the first five days of kinetic test lower concentration of drug was observed during antibiotic release from chemically modified granules against to concentration of gentamcin released from HAp only soaked in antibiotic. The immobilization method did not influence gentamicin antimicrobial properties.

Sterilization processes (UV radiation, ethylene dioxide, steam sterilization in autoclave) also did not damage antibiotic structure. Sterilized HAp-chitosan-gentamicin and HAp soaked in gentamicin granules inhibited bacterial growth just as non-sterilized granules. Those results confirmed our previous experiments [8,18]. Friess i Schlapp [2] also investigated the influence of sterilization on gentamicin activity and found that sterilization process did not change antimicrobial drug activity.

The possibility of various shapes of moulders forming is the next important advantage. We made different shapes of biocomposite, which structure was stabile in PBS.

CONCLUSION

Gentamicin-chitosan modified HAp has antimicrobial activity against Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli and its surface is resistant to biofilm formation. Antibiotic is attached strongly, its elution from HAp is slow and different amounts of gentamicin could be immobilized on HAp-chitosan surface. Moreover sterilization process does not influence biomaterial antimicrobial properties. HAp-chitosan mass plasticity is a very important feature, because we could make various moulders depending on the requirements.

The conclusion was that HAp-chitosan moulders could be potentially applied in orthopaedic and craniofacial surgery.

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SUMMARY

Forming a biofilm on orthopedic implant surface is a serious problem nowadays. This research was focused on making new type of biocomposite with antimicrobial properties. Biocomposite was protected from bacterial adhesion (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25992) and biofilm formation. Biomaterial hybrid showed prolonged antimicrobial activity (25 – 41 days). HAp-chitosan-gentamicin granules were resistant to sterilization process and saved its antimicrobial activity. The possibility of forming different shapes from chitosan-modified HAp granules make this biocomposite promising for future medical applications.

Keywords: hydroxyapatite, chitosan, antibiotic immobilization, implant infection, gentamicin

STRESZCZENIE

Tworzenie biofilmu na powierzchni implantów ortopedycznych stanowi poważny problem w obecnych czasach. Badania przedstawione w tej pracy dotyczyły opracowaniu nowego typu biomateriału posiadającego właściwości przeciwbakteryjne.

Kompozyt był chroniony przed adhezją bakterii (*Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25992) i wytworzeniem przez nie biofilmu. Hybrydowy biomateriał HAp-chitosan-gentamycyna wykazywał przedłużoną aktywność przeciwbakteryjną (25-40 dni). Ponadto granule HAp-chitosan-gentamycyna były odporne na proces sterylizacji i zachowały swoją antybakteryjną aktywność. Łatwość formowania różnych kształtek z granul hydroksyapatytowo-chitosanowych czyni ten biokompozyt obiecującym biomateriałem dla przyszłych zastosowań medycznych.

Słowa kluczowe: hydroksyapatyt, chitosan, immobilizacja antybiotyków, zakażenia implantów, gentamycyna