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COMPOSITES THEORY AND PRACTICE

Magdalena Ziąbka[1], Anna Mertas[2], Wojciech Król[2]

[1] AGH - University of Science and Technology, Department of Ceramics and Refractory Materials al. Mickiewicza 30, 30-059 Krakow, Poland
[2] Chair and Department of Microbiology and Immunology - Medical University of Silesia, The School of Medicine and Division of Dentistry in Zabrze, ul. Jordana 19, 41-808 Zabrze - Rokitnica, Poland
**Corresponding author. E-mail: ziabka@agh.edu.pl*

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POLY(LACTIDE-CO-GLYCOLIDE) COMPOSITES CONTAINING ANTIBACTERIAL SILVER NANOPARTICLES - *IN VITRO* PRELIMINARY STUDY

This work concerns the biological and morphological assessment of poly(lactide-co-glycolide)/silver nanoparticle (nAg) composites prepared by the slip-casting method. Due to the significance of the bactericidal properties of such materials, antibacterial activity against Gram-positive - *Staphylococcus aureus* and Gram-negative - *Escherichia coli* was evaluated by means of the surface deposition method. By inductively coupled plasma mass spectrometry (ICP-MS), it was possible to evaluate the amount of released silver ions and to determine their impact on the surrounding environment of bacteria. The material microstructure and dispersion of the modifier phase were estimated by using electron scanning microscopy with elemental analysis in micro-areas (SEM+EDS). The roughness and Theta angle measurements allowed us to define the surface character of the investigated materials. The tests of antibacterial efficacy proved that nanosilver-modified composites have bactericidal activity against the tested bacteria. The antibacterial efficacy of the tested materials depends on the amount of modifier phase (nAg). Along with an increasing volume fraction of modification phase, a different degree of the homogenization process was observed as well as a reduction in composite homogenization, a roughness increase and Theta angle decrease. At the same time, the composites showed higher wettability. Spectrometric analysis showed that the amount of released silver ions depends on the amount of nanoparticles present in the polymer matrix.

Keywords: poly(lactide-co-glycolide), nanocomposites, nanosilver, bactericidal properties

KOMPOZYTY POLILAKTYD-KO-GLIKOLID ZAWIERAJĄCE ANTYBAKTERYJNE NANOCZĄSTKI SREBRA - WSTĘPNE BADANIA *IN VITRO*

W pracy przedstawiono ocenę morfologiczną i biologiczną kompozytów polilaktyd-ko-glikolid/nanocząstki srebra (nAg) otrzymanych w procesie odlewania folii. Materiały kompozytowe oraz polimer w czystej postaci poddane zostały testom oceny aktywności przeciwbakteryjnej. Skuteczność bakteriobójcza została oceniona wobec wzorcowych szczepów Gram-dodatnich bakterii *Staphylococcus aureus* oraz Gram-ujemnych bakterii *Escherichia coli,* wykorzystując w tym celu technikę osadzania powierzchniowego. Za pomocą techniki elektronowej mikroskopii skaningowej (SEM) oraz spektroskopii z dyspersją energii (EDS) przeprowadzono ocenę mikrostruktury oraz stopnia dyspersji fazy modyfikującej w matrycy polimerowej. Dzięki przeprowadzonym pomiarom profilometrycznym oraz kąta zwilżania określono charakter powierzchni badanych materiałów. Ilość uwolnionych jonów srebra oznaczono techniką spektrometrii mas ze wzbudzeniem w plazmie indukcyjnie sprzężonej (ICP-MS). Antybakteryjne działanie materiałów korelowano z ilością uwolnionych do otoczenia jonów srebra. Na podstawie wyników przeprowadzonych testów i obserwacji wykazano, że kompozyty polimerowe modyfikowane nanosrebrem wykazują działanie bakteriobójcze wobec wzorcowych szczepów Gram-dodatnich bakterii *Staphylococcus aureus* oraz Gram-ujemnych bakterii *Escherichia coli*. Skuteczność przeciwbakteryjnego działania badanych materiałów kompozytowych uzależniona była od ilości dodatku antybakteryjnego (nAg). Wraz z rosnącym udziałem fazy modyfikującej obserwowano niższy stopień homogenizacji kompozytów, wzrost chropowatości i spadek kąta zwilżania, ale tym samym kompozyty wykazywały wyższą zwilżalność. Największą skutecznością działania przeciwbakteryjnego wobec bakterii Gram-dodatnich i Gram-ujemnych wykazywały się kompozyty z dodatkiem trzech procent wagowych nanosrebra.

Słowa kluczowe: polilaktyd-ko-glikolid, nanokompozyty, nanosrebro, właściwości bakteriobójcze

INTRODUCTION

Polymers such as polyglycolide and its copolymers with lactide, due to their biocompatibility and relatively good mechanical properties, are more often used in medical applications as very good materials for bone fixation, such as biodegradable implants in orthopedic applications, bioresorbable bone plates and screws for the internal fixation of bone fractures, fillers for bone defects and a base for guided tissue regeneration - scaffolds for bone repair and microspheres for drug delivery systems [1, 2]. The main advantage of such

materials is their degradation process through the hydrolysis of their ester bonds into compounds naturally present in the organism which are eventually resorbed, e.g. removed from the body by normal metabolic pathways [3]. These include no additional removal operations after healing of the tissue; no long-term risks caused by a permanent implant inside the human body; and no interferences with diagnostic instruments such as magnetic resonance imaging (MRI) or X-ray imaging. However, pure polymers usually lead to adverse clinical effects such as inflammatory or allergic reactions caused by the acidic monomers degraded from polymers. The incorporation of biocompatible fillers into the bioresorbable polymer matrix may provide an alternative to reduce or eliminate inflammatory or allergic reactions. Because of their excellent tissue response, osteoconductivity and bioactive properties, hydroxyapatite (HA) and tricalcium phosphate (TCP) have been extensively studied as fillers to be incorporated into resorbable poly-hydroxy acids, namely polyglycolide, polylactides, and their copolymers [4]. These kinds of materials are also used to cover an implant surface to improve integration between the bone and the implant, as well as for the preparation of the base for tissue engineering. To reduce the bacterial infection of implanted medical devices, silver nanoparticles (nAg) are incorporated into biomaterials [5-7]. It has been recently demonstrated that loading PLLA or PLGA with silver nanoparticles greatly reduces bacterial growth [8, 9].

The characteristic properties of nAg are: very small size, large surface area and unique physicochemical properties, which make silver antibacterial. Among various nanomaterials like copper, zinc, titanium, magnesium and gold, nAg demonstrates the highest bactericidal efficacy against bacteria, viruses and other eukaryotic microorganisms [10]. One of the first documented reports describing the use of silver for medical treatment dates to ancient times [11]. Even then, this metal was widely used for preventing the spread of diseases.

The mechanism responsible for the bactericidal activity of nAg has not been fully explained yet. However, their bactericidal efficacy is observed to originate in the absorption of free silver ions and then with the interruption of ATP (adenosine triphosphate), as well as DNA replication, the creation of reactive oxygen species (ROS), and also direct cell membrane damage [12].

Silver in the metallic form is neutral, but in contact with moisture and oxygen it is ionized. Silver ions exhibit very high reactivity, react with the thiol groups (-SH) of bacteria and viruses, remove hydrogen atoms and form a disulfide bridge (-SS-), and thus a bacterium loses its ability to breathe [13]. Silver ions also cause damage to the cell wall, a complex formed between silver ions and proteins may interfere with the metabolism of bacterial cells [14]. In addition, silver ions can interact with the DNA and RNA of bacteria by denaturation and inhibiting bacterial replication [15]. The bactericidal efficacy of silver is varied in the case of different forms of bacteria. The effectiveness of antibacterial efficiency is mainly due to the construction of the bacterial cell wall, and also depends on the amount of released silver ions to the environment. Stronger bactericidal activity is usually observed for Gram-positive bacteria for which peptidoglycan determines 50÷90% of the cell wall components (only one layer protects the cytoplasm). The cell wall of Gram-negative bacteria is characterized by a more complicated construction - both structurally and chemically. For Gram-negative bacteria, a thin peptidoglycan layer (5÷20% of the cell wall components) is located between the outer membrane and cytoplasmic membrane (cytoplasm is protected by two layers). In addition, the outer membrane of the cell wall contains lipopolysaccharide, which supports cell-mediated immunity [16-19].

Besides the bactericidal function of nanosilver discussed in the literature, there are many factors that can cause an undesirable cytotoxicity effect of nanomaterials. The toxic response of materials may be influenced by the quantity of nanoparticles, their size, shape and amount of ions released into the environment. Upon entering the body, silver is absorbed through the digestive tract system, skin and mucous membranes and retained in the reticuloendothelial system, responsible for maintaining infectious resistance, the removal of abnormal or dead cells and unwanted substances from the body. When binding with collagen and other fibrous structures such as the lamina epithelium, endothelium, muscle sheaths, ligaments and nerves, silver can cause toxic reactions [20].

The reason for silver's cytotoxicity can also be found in the formation of ROS which damages cellular DNA and RNA by the process of apoptosis. Due to the strong oxidation-reduction function (redox) of precious metals, silver nanoparticles increase the ROS-concentration and induce oxidation stress in cells, initiating the peroxidation process, which causes deformation of the cell membrane structure, followed by depolarization and inhibition of the membrane enzymes activity. This leads to a loss in the integrity of the cell membrane and causes disorder in the oxidative phosphorylation in mitochondria [21], which in consequence, contributes to cell degradation, leads to numerous diseases and accelerates body ageing [22]. The higher the concentration and larger size of nanoparticles, the stronger the cytotoxic effect [23]. Commercially available colloidal silver, metallic silver or silver nanoparticles are used for burn treatment, ulcerations (bandages, dressings, sutures), the production of different types of dental and medical equipment (like prostheses), bioresorbable fibers, even textiles. It is also used as coatings for stainless steel materials, for sunscreen components and water treatment. Finally, it is added as a component of detergents used for cleaning hospital surface areas. Nanosilver is also used as a bactericidal filler for biostable, biodegradable and bioresorbable polymers [24-26].

In the case of polymer composites with silver nanoparticles, the amount of silver ions released into the environment depends mainly on the type of matrix and the introduction of nanoparticles into the polymer matrix. In the case of bioresorbable polymers such as polylactide (PLA) or poly(lactide-co-glycolide) (PGLA), the amount of silver ions released will increase depending on the immersion time and the degree of polymer degradation. Therefore, it seems essential to select the nanoadditive volume fraction properly. A too low amount of modifier may not be sufficient to eliminate bacteria, while a too high amount may cause cytotoxic effects.

In this study, silver nanoparticles were incorporated into a biodegradable poly(lactide-co-glycolide) matrix by the slip-casting method. PGLA and PGLA/Ag composites with different contents of silver nanopowder were obtained in the form of foils. The aim of this work was to determine the optimal amount of silver nanoparticles in poly(lactide-co-glycolide) nanosilver (PGLA/Ag) composites to obtain materials with bactericidal activity. The modification of a resorbable polymer matrix with bactericidal silver nanoparticles which might be proposed as an external covering of polymer implants would be considered as a new approach. Thanks to the bactericidal additions to the external layer, silver ions will be systematically released after introduction to the body for a known period of time, which will be the consequence of polymer resorption. Silver ions will stimulate tissue for faster regeneration and a better healing process.

Such a modification of the polymer external layer achieved by their immersion in a solution containing silver nanoparticles will not only improve the "implant-tissue" integration process but in consequence will also reduce the time and costs of hospitalization.

The morphology of the PGLA/Ag nanocomposites was observed using scanning electron microscopy (SEM) with EDS analysis. The surface properties such as wettability and roughness of these specimens were investigated. The release of silver ions was determined during 10 weeks of sample immersion in a UHQ H_2O solution. Antimicrobial tests were carried out using *Staphyloccocus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. The current research shows that PGLA/Ag composites posess antibacterial efficacy against the employed standard bacteria strains.

MATERIALS AND METHODS

Poly(lactide-co-glycolide) (PGLA) containing 85% lactide and 15% glycolide units (Mn = 100000, η_{inh} = 1.85) produced at the Centre of Polymer and Carbon Materials PAN in Zabrze (Poland) was used in this work. The silver nanopowder was obtained from the Sigma-Aldrich Company (Mw = 107.87; particle size < 100 nm). Commercially available dichloromethane (CH_2Cl_2, POCH) and nitric acid (HNO_3, POCH) were also used.

Preparation of PGLA/Ag nanocomposites

The PGLA and PGLA/Ag composites were prepared in the form of a foil by the slip-casting method (Fig. 1). For that purpose, PGLA was dissolved in a solvent of dichloromethane. Silver nanopowder was incorporated into the polymer solution in the amount of 1.5 wt.% and 3 wt.%. The PGLA/Ag solution was stirred for 30 minutes by using ultrasonic waves to reduce silver agglomeration and then cast in a Petri dish. To evaporate the solvent, the foils were dried in a vacuum for 48 h.

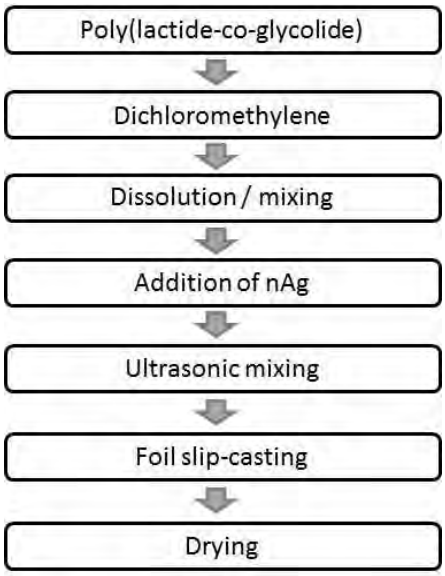


Fig. 1. Sample preparation

Rys. 1. Otrzymywanie próbek

Morphology investigations

Scanning electron microscope (SEM, Nova Nano SEM 200, FEI Company) images with an attachment for the chemical analysis of specimens in microareas with energy dispersive X-ray spectroscopy (EDX, EDAX) were obtained. The experiment was carried out in low vacuum conditions in the secondary electron mode. The samples were covered with a carbon layer.

Contact angle

The contact angles were determined by using an automatic system, Drop Shape Analysis DSA 10 (Kruss) with water drops on flat specimen surfaces. For each sample, ten measurements were taken. These droplets were analyzed using a camera and transferred to a computer for angle measurement. As the wetting agent, UHQ water was used. The contact angle of the PGLA and PGLA/Ag foils was measured before and after 10 weeks of incubation.

Surface roughness

The surface roughness (*Ra*) was measured for three samples per group, rectangular shape (0.25 mm in thickness) on a L = 48 mm sample path, using profilometry (T-500, Hommelwerke) surface topography.

Silver ion release

The PGLA and PGLA/Ag composite foils were cut into 1 cm x 3 cm pieces, which weighed approximately 10 mg and were 0.25 mm in thickness. The samples were incubated at 37 ±1°C in 30 ml of UHQ water for 10 weeks. In order to protect the silver ions (Ag^+) against reduction into metallic silver and to perform ICP-MS analysis, the filtered samples were acidified with nitric acid up to the final concentration of 0.1 mol/L. The *in vitro* release of silver ions was carried out by using plasma atomic emission spectrometry (ICP-MS). In the ICP-MS measurements, the Ag107 isotope was used for total chromium determination using an external standard calibration procedure.

Plasma sterilization

Quadratic PGLA and PGLA/Ag foils (10 mm x10 mm) were sterilized by using low temperature plasma. The specimens were put into a sterilization chamber (Sterrad 120 apparatus). Hydrogen peroxide vapour was used in a double cycle (2 x 45 minutes).

Antibacterial test

The *in vitro* antibacterial activities of the pure polymer and PGLA modified with silver nanoparticles after sterilization were examined according the method by Xiaoyi Xu et al. [8]. Five samples per group were analyzed. The following microorganisms were used: Gram-positive *Staphylococcus aureus* ATCC 25923 and Gram-negative *Escherichia coli* ATCC 25922 standard bacteria strains. The following samples PGLA, PGLA/1.5 wt.% Ag and PGLA/3 wt.% Ag were incorporated into the bacterial suspensions in triptonic water, which contains about $1.5x10^5$ colony forming units (CFU) of *Staphylococcus aureus* or *Escherichia coli*, respectively. The mixtures were incubated at 37°C in static conditions for 9 h. Triptonic water with only *Staphylococcus aureus* or *Escherichia coli* was tested as a blank control and polymer samples in triptonic water without bacteria and pure triptonic water were tested as a negative control. After incubation, 20 µL of each sample was seeded onto an agar plate supplemented with a 5% addition of ram's blood. During the experiment the surface spread plate technique was used. The agar plates were incubated at 37°C for 24 h. Then the numbers of bacterial colonies (CFU) were counted. The following equation: ABE [%] = (Vc – Vt) / Vc x 100 was used to investigate the antibacterial efficacy of the samples. The numbers of viable bacterial colonies of the blank control were represented by Vc and Vt stood for the test specimen.

RESULTS AND DISCUSSION

Morphology investigations

Figures 2 and 3 present the SEM images of pure PGLA and PGLA/Ag composites containing 1.5 and 3 wt.% silver. The sample microstructures were observed at the beginning and after 10 weeks of incubation in UHQ H_2O. According to these photographs, the silver particles were not well distributed in the polymer matrix and phase separation occurred.

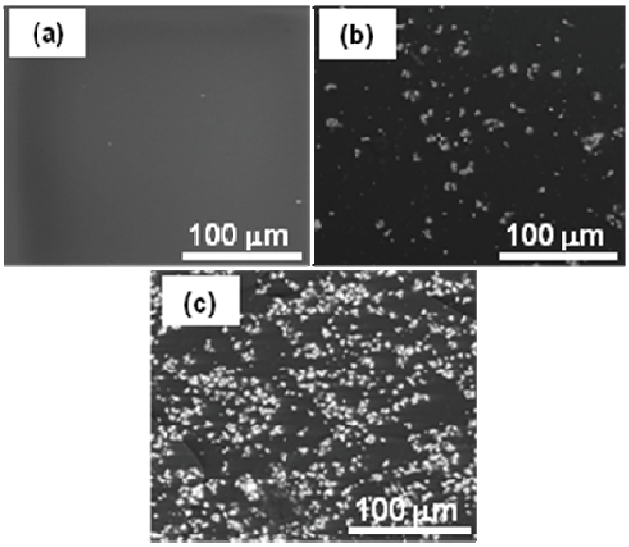


Fig. 2. SEM image of pure PGLA (a), composite surface of (b) PGLA/1.5 wt.% Ag and (c) PGLA/3 wt.% Ag

Rys. 2. Zdjęcie SEM powierzchni czystego PGLA (a), kompozytu (b) PGLA/1,5% wag. Ag i (c) PGLA/3% wag. Ag

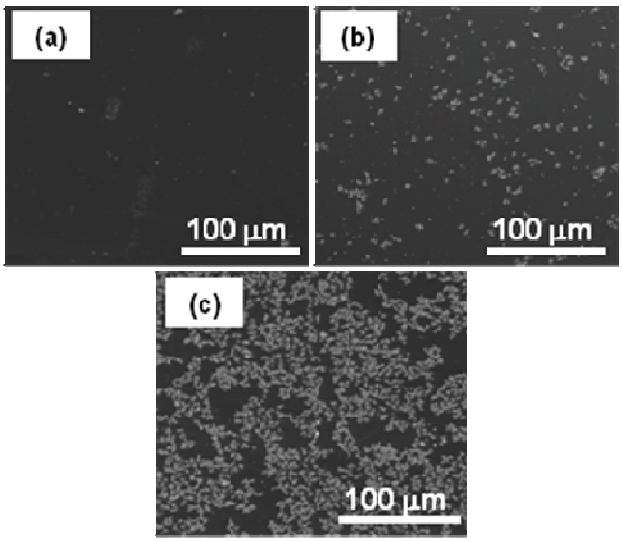


Fig. 3. SEM image after incubation in UHQ water solution of pure PGLA (a), composite surface of (b) PGLA/1.5 wt.% Ag and (c) PGLA/3 wt.% Ag

Rys. 3. Zdjęcie SEM powierzchni czystego PGLA (a), kompozytu (b) PGLA/1,5% wag. Ag i c) PGLA/3% wag. Ag po inkubacji w wodzie destylowanej o ultrawysokiej czystości

The particles were also agglomerated into large, non-uniformly shaped aggregates. Aggregates with diameter ranges between 5 and 15 μm are shown in Figure 4a. After 10 weeks of incubation there were more nanoparticles exposed on the polymer surface. This behavior is related to the degradation process of the polymer matrix and gradual silver exposure, which was proved by roughness measurements.

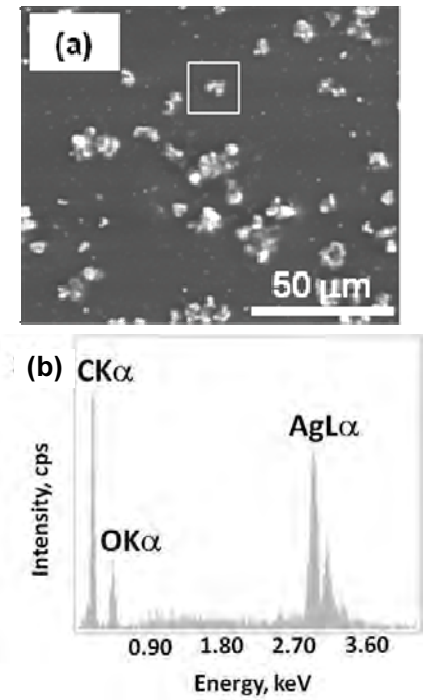


Fig. 4. SEM image (a) and EDS analysis in selected area (b) of PGLA/3 wt.% Ag after incubation in UHQ water solution

Rys. 4. Zdjęcie SEM (a) i analiza EDS w wybranym obszarze (b) kompozytu PGLA/3% wag. Ag po inkubacji w wodzie destylowanej o ultrawysokiej czystości

Contact angle

The results received for the PGLA and PGLA/Ag foils presented in Figure 5 showed that an addition of silver nanopowder caused a decrease in the contact angle. A silver addition of 3 wt.% reduced it by 13%, whereas 1.5 wt.% of the same additive did not influence this parameter.

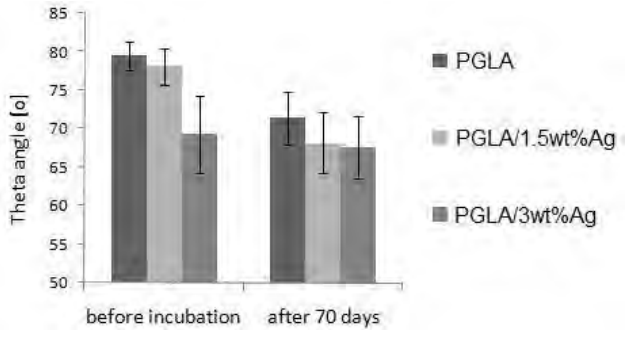


Fig. 5. Contact angle of pure PGLA and PGLA/Ag composite samples before and after incubation in water solution

Rys. 5. Kąt zwilżania PGLA oraz kompozytów PGLA/Ag przed i po inkubacji w wodzie destylowanej

After 10 weeks of incubation in a water solution, the contact angle decreased in all the investigated samples. Such results might be caused by the silver surface area (5 m^2/g) and its tendency to agglomeration. Non-uniform silver distribution and polymer resorption is also responsible for the higher wettability.

The incubation time had an influence on the surface topography. The rougher the surface was, the lower the contact angle and higher wettability observed. For the PGLA/3 wt.% Ag samples, the theta angle decreased insignificantly. The hydrophilic surface positively influenced not only cell adhesion and its proliferation but also was responsible for bacteria adhering. The incorporation of silver nanoparticles into a hydrophilic polymer matrix seems to be appropriate for an ideal connection, whose main aim is to catch and deactivate bacteria.

Roughness tests

Roughness tests were carried out on pure polymer and composite foils. The measurements were done before and after 10 weeks of incubation in a water solution (Fig. 6). For the initial samples, a 3 wt.% silver additive caused a roughness increase (from 70 μm for the PGLA foil up to 170 μm for the composite foil). The roughness differences were also clear in the line profiles (Fig. 7). After immersion, significant differences in the topography were observed for all the investigated specimens. The more nanoparticles incorporated into polymer matrix, the rougher the sample surface became, which could be easier noticed after their incubation. It was not only connected with the amount of silver addition but also with rapid polymer resorption. During the dissolving of the PGLA matrix, the material surface became rougher and the silver particles were exposed.

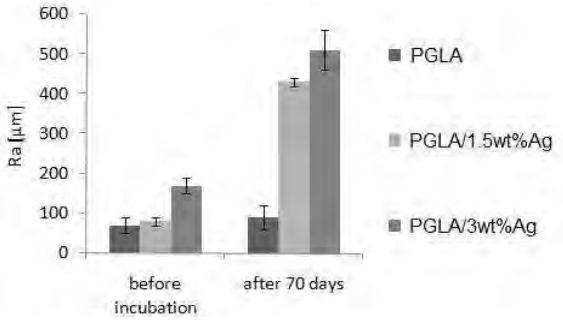
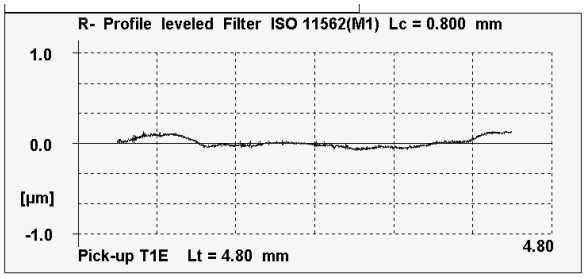


Fig. 6. Roughness of pure PGLA and PGLA/Ag composite samples before and after incubation in water solution

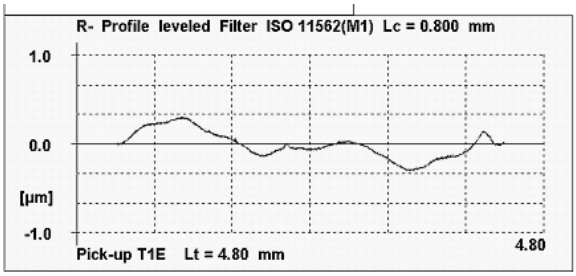
Rys. 6. Chropowatość PGLA oraz kompozytów PGLA/Ag przed i po inkubacji w wodzie destylowanej

From the implantology and tissue engineering point of view, high biomaterial roughness is not always favorable. It might create excellent conditions for biofilm formation instead of protein adsorption. Introducing silver nanoparticles into the polymer matrix increases the material roughness but on the other hand, it also reduces the amount of bacteria.

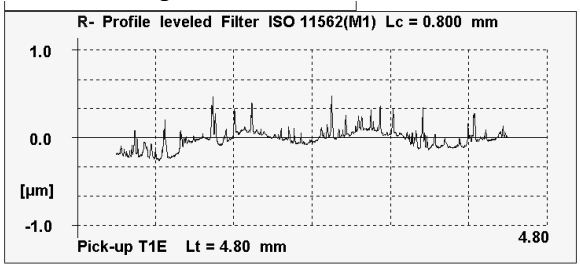
PGLA before incubation



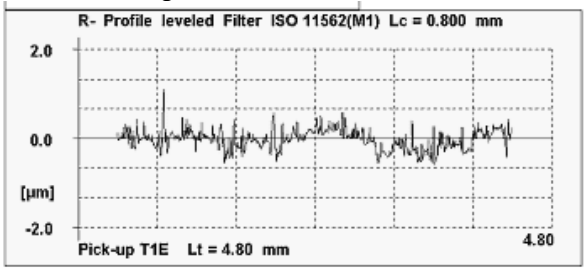
PGLA after 10 weeks incubation



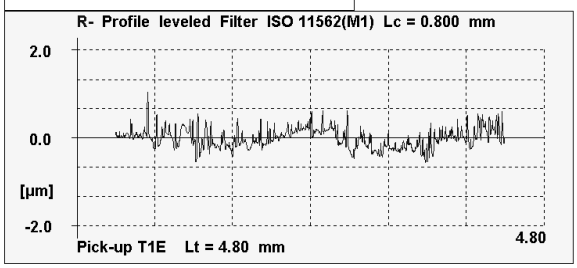
PGLA/1.5wt%Ag before incubation



PGLA/1.5wt%Ag after 10 weeks incubation



PGLA/3wt%Ag before incubation



PGLA/3wt%Ag after 10 weeks incubation

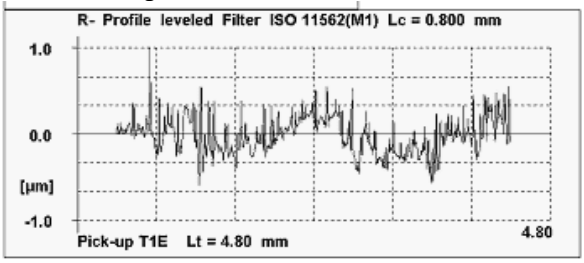


Fig. 7. Profile diagrams of pure PGLA and PGLA/Ag composites before and after 10 weeks incubation in water solution

Rys. 7. Profile powierzchni dla PGLA oraz kompozytów PGLA/Ag przed i po 10 tygodniach inkubacji w wodzie destylowanej

Silver ion release

The PGLA specimens modified with 1.5 wt.% and 3 wt.% silver particles, release silver ions after contact with the UHQ water solution. During immersion, the metallic silver nanoparticles were oxidized and converted into silver ions (Ag^+). Figure 8 shows the concentration of Ag+ detected in the solutions where the PLGA/Ag nanocomposites with 1.5 or 3 wt.% nanoparticles, respectively, were incubated as a function of time. For both nanocomposite samples, the amount of detected Ag+ was absolutely dependent on the silver loading. Moreover, the release was relatively slow at the early stage of incubation and it became faster after 3 weeks of exposure to the water environment. The ICP measurements indicated that the amount of Ag+ detected increased with the incubation time, thus the rate of Ag+ release is enhanced by the polymer degradation. A similar behavior was observed for both composites. For the PGLA/3 wt.% Ag, the amount of detected Ag+ after 10 weeks of immersion was equal to 2.030 mg/dm^3, which was around five times higher than for the PGLA/1.5 wt.% Ag. Such a result is connected with the amount of silver filler, and its influence on polymer degradation. The more nanoparticles incorporated, the faster the degradation and the larger quantities of silver ions observed. The initial content of silver nanopowder is responsible for the faster release of Ag^+.

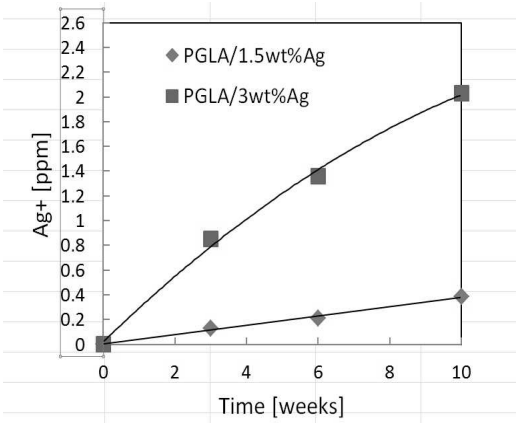


Fig. 8. Ag^+ ions release during 10 weeks of sample immersion in UHQ water

Rys. 8. Uwalnianie jonów srebra podczas 10-tygodniowej immersji próbek w ultraczystej wodzie destylowanej

Antibacterial activity

The antibacterial tests showed that composites modified with silver nanoparticles exhibit antibacterial efficacy (ABE). Such an effect is important from the

clinical point of view. Table 1 shows that the addition of 1.5 wt.% silver to the PGLA matrix is enough to kill bacteria after 9 hours of incubation. ABE against *Escherichia coli* is lower than against *Staphyloccocus aureus*. The difference is not high and according to [24, 27] can be connected with the bacteria cell walls. *Eschericha coli* belong to Gram-negative bacteria with a negative charge that is why its surface is attracted by ions with an opposite charge which helps bacteria in the approach to polymer surfaces.

TABLE 1. **Antibacterial efficacy of PGLA and PGLA/Ag composites against *Staphylococcus aureus* and *Escherichia coli* standard strains**

TABELA 1. **Skuteczność bakteriobójcza PGLA i kompozytów PGLA/Ag przeciw *Staphylococcus aureus* i *Escherichia coli***

Material	*Staphylococcus aureus* ATCC 25923		*Escherichia coli* ATCC 25922	
	(initial density of bacterial suspensions was 1.5 x 10^5 CFU/ml)			
	CFU/ml after 9 hours incubation with material	Antibacterial efficacy ABE [%]	CFU/ml after 9 hours incubation with material	Antibacterial efficacy ABE [%]
PGLA	3.2 x 10^8	0	3.5 x 10^8	0
PGLA/1.5 wt.% Ag	0	100	3.5 x 10^4	99.9
PGLA/3 wt.% Ag	2.4 x 10^3	99.9	1.0 x 10^3	99.9
BLANK	3.2 x 10^8	---	3.5 x 10^8	---

CONCLUSIONS

Silver nanoparticles show effective bactericidal properties against bacteria, viruses and candidias, that is why they are willingly applied in various antimicroorganism therapies. Innovative applications of silver nanoparticles not only reduce hospital-acquired infections, but also improve the quality of life and well-being of the patients that result from a reduction in the post-operative infection rate. It is forecasted that in the future, nanosilver will be applied in various medical fields as an antibacterial factor. Due to their unique properties, nanomaterials play an important role in the development of contemporary disease treatment methods. However, clinical use must be preceded by verification and examination of the impact of the nanomaterials on living organisms. By using the slip-casting method, it is possible to obtain PGLA and PGLA/Ag composites. The ICP results showed that composites with higher concentrations of silver nanoparticles possess a higher rate of silver ion release. The rate of Ag+ release is enhanced by the polymer degradation and increased with the incubation time. The antimicrobial tests proved that the composites prevent the growth of *Staphylococcus aureus* and *Escherichia coli*. The introduction of 1.5 wt.% silver filler into the PGLA matrix is enough to kill bacteria after 9 hours of incubation.

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