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The effect of polyhexamethylene guanidine hydrochloride on biofilm formation on polylactide and polyhydroxybutyrate composites

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ABSTRACT

The aim of this study was to investigate the impact of polyhexamethylene guanidine hydrochloride (PHMG) derivatives introduced into polyhydroxybutyrate (PHB) and polylactide (PLA) on biofilm formation by *Escherichia coli* and *Staphylococcus aureus* on the surface of these composites. We also determined bactericidal activity of the applied PHMG derivatives according to standard ISO 22196. The composites were made of PLA and PHB containing PHMG stearate, PHMG granular polyethylene wax, and PHMG salt of sulfanilic acid at concentrations ranging from 0.2 to 1.0 % (w:w). The results indicate that PLA and PHB enriched with PHMG stearate and salt of sulfanic acid do not have bactericidal properties ($R < 2$) while PLA and PHB enriched with PHMG granular polyethylene wax have bactericidal properties ($R > 2$). Increased concentration of PHMG derivatives improved bactericidal activity of the investigated composites thus inhibiting biofilm formation on their surface. The composite containing PHMG granular polyethylene wax was the strongest inhibitor of biofilm formation by *S. aureus*.

Introduction

Produced from lactic acid, polylactide (PLA) is considered one of the most promising biodegradable polymers of the 21st century. Owing to its good physicochemical properties and biodegradability, it is applied in many areas including medicine and commercial packaging (Żakowska, 2002). Moreover, due to the need to obtain plants for its production, it can contribute to the global agricultural growth. Biodegradable packaging materials produced from PLA, especially for use in the food industry must have high mechanical resistance as well as barrier and biocidal properties (Reid, 1999; Dogan, 2009; Richert el al. 2013; Hoffman et al., 2005). One way of reducing microbial growth and preventing biofilm formation is introducing into the polymer bactericidal substances such as sorbic acid, triclosan, grapefruit seed extract, bacteriocin (Malinowska-Pańczyk et al., 2010) or poly (hexamethylene guanidine) (PHMG) and its derivatives. Due to high thermal resistance, PHMG derivatives can be widely applied in the production of PLA polymer materials (Wyrębska et al., 2009; Królikowski et al., 2009; Richert and Walczak, 2012). When enriched with bactericidal substances, composite packaging materials can inhibit the growth of harmful microorganisms, reducing the risk of contaminating food products, and thus extending their shelf life (Appendini and Hotchkis, 2002; Kim et al., 2009; Wei et al., 2009; Zhou et al., 2009).

Another polymer, polyhydroxybutyrate (PHB), produced by microorganisms, (eg. *Alcaligenes eutrophus, Bacillus megaterium, E. coli, Nocardia, Pseudomonas, Rhizobium*) has properties typical of thermoplastics (Bucci et al., 2005; Stachurek, 2012). Obtained in the glucose fermentation, PHB is applied in medicine as a drug delivery carrier and in tissue engineering as a scaffold material (Zinn et al., 2001). It is also used in cosmetic packaging.

The aim of this study was to investigate bactericidal properties of polylactide and polyhydroxybutyrate composites containing three different PHMG derivatives and to evaluate biofilm formation on their surface.

Materials and methods

Materials

For the production of the investigated composites we used biodegradable PLA and PHB polymers and the following

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bactericidal PHMG derivatives: PHMG granular polyethylene wax, PHMG salt of sulfanilic acid PHMG stearate. The composite films were prepared in the Institute for Engineering of Polymer Materials and Dyes (Poland). They are composed of PLA or PHB polymers and copolymers (PHMG + organic carriers: polyethylene wax, sulfanilic acid, and stearate). PHMG derivatives were prepared according to the Patent No: P.388062, 2009 (Królikowski et al., 2009).

Preparation of film samples

The pelleted mixture of PLA and PHB with PHMG derivatives was prepared using the co-rotating twin-screw extruder type BTSK 20 (screw diameter Ľ 20 mm, L/D Ľ 40) with a segmented plasticizing system (Bühler, Germany). Pelletizing was performed in the form of a cool extrudate in the air with the temperature of 25 ± 3 °C. The extruded pellets had the following contents of PHMG derivatives: 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0% (wt). They were processed into films using a single-screw extruder type PlastiCorder PLV 151 (Brabender, Germany) with a screw diameter of 19.5 mm and L/D Ľ 25.

The following symbols of the composites were used in the tests: A-PHB/PLA (PHB or PLA with the PHMG salt of sulfanilic acid), W-PHB/PLA (PHB or PLA with PHMG granular polyethylene wax), S-PHB/PLA (PHB or PLA with PHMG stearate). Control samples were pure PLA or PHB without PHMG.

The evaluation of bactericidal activity of the composite films

We evaluated bactericidal properties of PLA and PHB films containing PHMG derivatives according to standard ISO 22196. The study is based on two bacterial strains, i.e. *Staphylococcus aureus* (ATCC 6538P) and *E. coli* (ATCC 8739). Bacterial strains were grown on nutrient broth containing [g/dm^3] peptone – 5.0, meat extract – 3.0 with pH – 7.4. Bacteria were incubated at 37 °C for 24 h. From each culture 1 cm^3 suspension was transferred with a sterile pipette into Eppendorf tubes, which were then centrifuged at 10 000 rpm. After removing the supernatant the precipitate was suspended in 1 cm^3 of Nutrient Broth (1/500 NB) containing [g/dm^3] meat extract – 0.006, peptone – 0.02, sodium chloride – 0.01, distilled water – 1 dm^3 with pH 6.8–7.2. The obtained bacterial suspension was transferred to a densitometer (Densi-La-Meter® II, Erba Lachema, Czech Republic) for the measurement of its optical density, subsequently brought to a value of 0.5, which, according to McFarland standards, corresponds to 1.5 × 10^8 bacterial cells in 1 cm^3. The suspension was then diluted with Nutrient Broth (1/500 NB) until the number of bacterial cells was 7.5 × 10^5 1 cm^3. The final suspensions were transferred to film samples (5 × 5 cm) and covered with sterile glass slides (4 × 4 cm) in order to evenly distribute microorganisms on the surface.

Subsequently, the samples were incubated at 35 °C for 24 h. After this time, live and viable bacterial cells were counted on the surface of the test and control samples. For this purpose the samples were rinsed with 10 cm^3 of SCDLP medium containing [g/dm^3] casein peptone – 17.0, soy peptone – 3.0, sodium chloride – 5.0, sodium dihydrogen phosphate – 2.5, glucose – 1.0, distilled water – 1 dm^3; pH 6.8–7.2. Diluted suspension was inoculated on the PCA medium containing [g/dm^3] yeast agar – 2.5, tryptone – 5.0, glucose – 1.0, agar – 15, distilled water – 1 dm^3 with pH 7.0–7.2. After 48-hour incubation at 35°C the numbers of grown colonies were converted to the numbers of bacterial cells.

Reduction in the number of viable cells by 2 orders of magnitude was interpreted as a bactericidal effect of the tested composite.

Reduction of the number of living and viable cells of tested bacteria (R) was calculated using the equation:

$$R = (U_t - U_o) - (A_t - U_o);$$

where:

U_o is the average of the common logarithm of the number of viable bacteria recovered from the control samples (PLA or PHB) immediately after inoculation (validation of recovery efficiency);

U_t is the average of the common logarithm of the number of viable bacteria recovered from the control samples (PLA or PHB) after 24 h (controls of survival in time, without PHMG derivatives);

A_t is the average of the common logarithm of the number of viable bacteria recovered from the test samples (W-PHB/PLA, A-PHB/PLA, S-PHB/PLA) after 24 h.

According to standard ISO 22196, the reduction of the number of cells capable of growth by two orders of magnitude ($R \geq 2$) was interpreted as a bactericidal effect of the investigated composite.

The evaluation of bacterial biofilm formation on the surface of PLA and PHB composite films

Fragments 0.5 × 1.5 cm of the control and test films (with and without PHMG derivatives) were sterilized with ethyl alcohol and placed in sterile Petri dishes with 10 cm^3 nutrient broth (composition [g/dm^3]: peptone – 5.0, meat extract – 3.0, pH 7.4). 10 ml suspensions of the appropriate bacterial strain with optical density of 1.0 according to Mc Farland standards were then transferred to Petri dishes and incubated at 35 °C for 48 h. Subsequently, bacterial biofilm formation on the surface of all film samples was assessed using spectrophotometric measurement of the adsorbed crystal violet. (Stepanovic et al., 2000; Sela et al., 2006; Kroupitski et al., 2009).

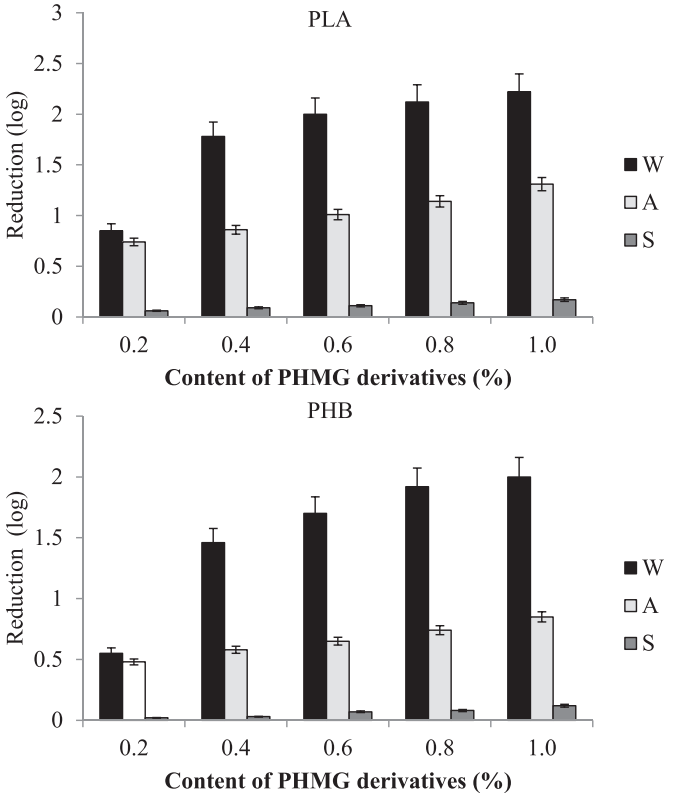

Fig. 1. Effect of PHMG derivatives introduced into PLA and PHB on *Escherichia coli* (W – PHMG granular polyethylen wax, A – PHMG salt of sulfanilic acid, S -PHMG stearate).