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Inhibitory Effect of Hemin – Poly(3-hydroxybutyrate) System on *E. coli* and *S. aureus* Growth

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The preparation method of porphyrin-polymer system – hemin-poly(3-hydroxybutyrate) (hemin-PHB) and its inhibitory effect on the growth of Gram-negative and Gram-positive microorganisms – Escherichia coli and Staphylococcus aureus, which are the common cause of wound infections, are presented. The assessment of antimicrobial activity was carried out by measuring of the inhibition zone diameters after bacterial daily incubation. It was shown that hemin-PHB efficiency was directly related to the hemin concentration. Thus, PHB containing 5% hemin (wt.%) inhibited E. coli and S. aureus significantly. The obtained porphyrin-polymer system can be recommended for use as the antiseptic material for wound treatment.

Keywords: Hemin, poly(3-hydroxybutyrate), Escherichia coli, Staphylococcus aureus, inhibitory effect.

Ингибирующее действие системы гемин – поли(3-гидроксибутират) на рост *E. coli* и *S. aureus*

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В работе описан метод получения порфирин-полимерной системы – гемин-поли(3-гидроксибутират) (гемин-ПГБ) и ее ингибирующее действие на рост грамотрицательных и грамположительных микроорганизмов – Escherichia coli и Staphylococcus aureus, которые являются частой причиной раневых инфекций. Оценка антимикробной активности проводилась путем измерения зоны задержки роста бактерий после их суточного инкубирования. Было показано, что эффективность гемин-ПГБ находилась в прямой зависимости от процентного содержания в полимере гемина. Так, существенное ингибирующее действие на E. coli и S. aureus оказывал ПГБ с 5% гемина (мас.%). Полученная порфирин-полимерная система может быть рекомендована для использования в качестве антисептического материала при обработке ран.

Ключевые слова: Гемин, поли(3-гидроксибутират), Escherichia coli, Staphylococcus aureus, ингибирующее действие.

Introduction

For many years, transition metal complexes, including compounds of natural and synthetic porphyrins, have been successfully used for various purposes and, in particular, in pharmacology as highly effective drugs.[1-7] For example, porphyrin containing compounds have been created and put into practice drugs for the treatment of malignant neoplasms, *e.g.* “Photogem”, “Tiosens” “Photofrin”, *etc*.[8-10]

One of the best known representatives of natural porphyrins is hemin, containing the ferric cation Fe^{3+} and the coordinating chloride anion Cl^-. Hemin was first obtained in 1853 by L.K. Teichmann by crystallization from blood.[11] Hemin is widely used in pharmacology to create drugs that correct heme deficiency in the body, *viz.* “Normosang” and “Pangematin”.[12-13] Also, there is evidence of the high effectiveness of hemin both in free form and as part of polymer matrices, with treatment of anemia, rheumatoid arthritis, cancer and in complex antibacterial therapy.[14-22] The antitumor and inhibitory effect of hemin is due to its ability to generate reactive oxygen species, thereby causing oxidative stress in malignant or bacterial cells.[17,19,20]

To increase the effectiveness of porphyrins, various approaches are used, in particular, their inclusion in a polymer carrier matrix. It should be noted that in recent years, special preference has been given to biocompatible and biodegradable polymers with minimal toxic effects.[23-26] Aliphatic polyesters, in particular, poly(3-hydroxybutyrate) (PHB), have the full required properties. PHB is produced by various microorganisms, such as the genera *Bacillus*, *Ralstonia* and *Rhizobium*. PHB is a stereoregular isotactic homopolymer of D(–)-3-hydroxybutyric acid with biodegradability.[27-30] Based on PHB, fibrous matrices containing Mn^{III}Cl-tetraphenylporphyrin (MnClTPP) and Fe^{III}Cl-tetraphenylporphyrin (FeClTPP) as modifying substances were created.[31,32]

This article describes a method for preparing the hemin-PHB system and its inhibitory effect against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) micro-organisms widespread in the environment. Such materials may be of interest as a prototype of a bactericidal patch to prevent bacterial infection of wounds of various etiologies. The relevance of the study is confirmed by the presence of a large number of publications devoted to the search for modern approaches to the creation of new generation antiseptic materials.[33-37]

Experimental

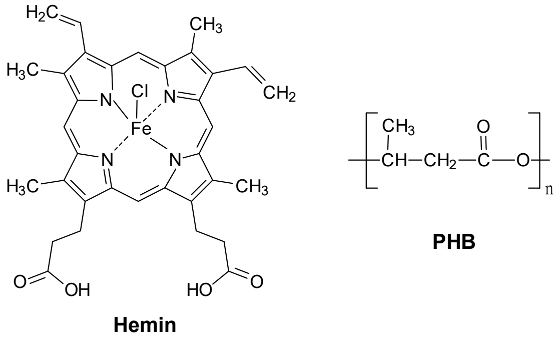
The materials used in the work were hemin (“Sigma-Aldrich”), poly(3-hydroxybutyrate) (“Biomer”), chloroform analytically pure (“Khimmed”), 99.0–99.4%, followed by distillation, sterile physiological solution (0.9% NaCl, “Khimikom”), meat peptone agar (MPA, “Khimikom”), industry turbidity standard for determining the total concentration of microorganisms (BAK-10 kit, “Art-Medica”). *Escherichia coli* (strain 1257) and *Staphylococcus aureus* (strain 209 P) cells were taken from the collection of cell cultures of the All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology. The structural formulas of hemin and PHB are presented in Figure 1.

To obtain the porphyrin-polymer system, solutions of hemin in chloroform (10^{-2} mol/L) were prepared. They were then added to solutions of PHB in chloroform at 60 °C using an automatic magnetic stirrer. The concentration of PHB in the solution was 7% (wt.), the content of hemin in the molding solution was 1, 3 and 5% (wt.) relative to the weight of PHB. Fibers were obtained by electrospinning[30-32] using a single-capillary laboratory setup with the following parameters: capillary diameter – 0.1 mm, electric voltage – 12 kV, distance between electrodes – 18 cm, electrical conductivity of the solution – 10 μS/cm. The sample was represented by a series of five samples of each composition.

The geometric parameters of fibrous materials were studied using a Hitachi TM-3000 scanning electron microscope (Japan) at an accelerating voltage of 20 kV. A layer of gold 10–20 nm thick was sputtered onto the surface of a sample of nonwoven fibrous material.

To obtain daily cultures of *E. coli* and *S. aureus*, they were reseeded in “Lamsystems” laminar and further cultivated on a slanted MPA in a thermostat (24 h, 37 °C, dry-air thermostat TV-80-1). Suspensions of 10^9 CFU/mL were prepared from daily cultures in sterile saline solution according to the turbidity standard. The obtained concentrations of suspensions were confirmed spectrophotometrically (λ = 600 nm, PE5400UF spectrophotometer, “Ekroskhim”). Next, successive dilutions were prepared from suspensions of daily cultures of *E. coli* and *S. aureus* (10^9 CFU/mL) in 10-fold increments: 10^8, 10^7, 10^6, 10^5 and 10^4 CFU/mL by titration in sterile saline solution. All dilutions were carried out in sterile tubes. To avoid foreign contamination, the tubes were sealed with sterile stoppers. Each dilution of *E. coli* and *S. aureus* was inoculated into Petri dishes with sterile MPA.

From samples of the polymer matrix hemin-PTB (hemin content - 1, 3 and 5% (wt.%) relative to the mass of PHB), round fragments with an area of 1 cm^2 were cut out with sterile scissors and placed in the center of the dishes with inoculations. After which they were incubated in a thermostat at 37 ºC for 24 h. The results were taken into account by the diameter of the growth inhibition zone (mm). The experiments were carried out in triplicate.


Hemin

PHB

Figure 1. Structures of hemin and poly(3-hydroxybutyrate).

Results and Discussion

The morphology of PHB-based fibrous materials with different hemin contents was determined using scanning electron microscopy. Figure 2 shows micrographs of nonwoven fibrous materials. The original polymer fibers are characterized by the presence of a large number of spindle-like thickenings (Figure 2a). The appearance of the thickenings is caused by the suboptimal electrical conductivity of the spinning solution (less than 1 μS/cm). The size of the thickenings in the transverse direction is 5–10 μm, in the longitudinal direction – 10–20 μm. The average diameter of the main fiber ranges from 1–3 μm. When hemin is added in an amount of 1%, the morphology of the fibrous material changes dramatically (Figure 2b,c,d). A large number of defective areas appear in the form of numerous adhesions, but the spindle-like thickenings partially remain. The average size of adhesions ranges from 50 to 200 μm. At the same time, the average diameter of the cylindrical sections of the fibers is practically unchanged and continues to remain at the level of 1–3 μm. The reason for the appearance of adhesions is not entirely clear, however, one of the explanations may be insufficient electrical conductivity and a decrease in the dynamic viscosity of the PHB molding solution when adding 1% hemin.

As can be seen in Figure 2c,d, with the addition of 3% and 5% hemin, spindle-like thickenings and adhesions on PHB fibers disappear almost completely. This is due, in our opinion, to an increase in the electrical conductivity of the molding solutions from 1 to 10 μS/cm. An increase in electrical conductivity leads to a uniform extension of a drop of the polymer molding solution into the field of electrostatic forces. A noticeable increase in electrical conductivity is associated with the appearance of metal complexes in the PHB solution. The average diameter of PHB fiber with 3–5% hemin ranges from 3–10 μm. The presence of thin and thick fibers indicates splitting of the primary jet of polymer solution during the electrospinning process.

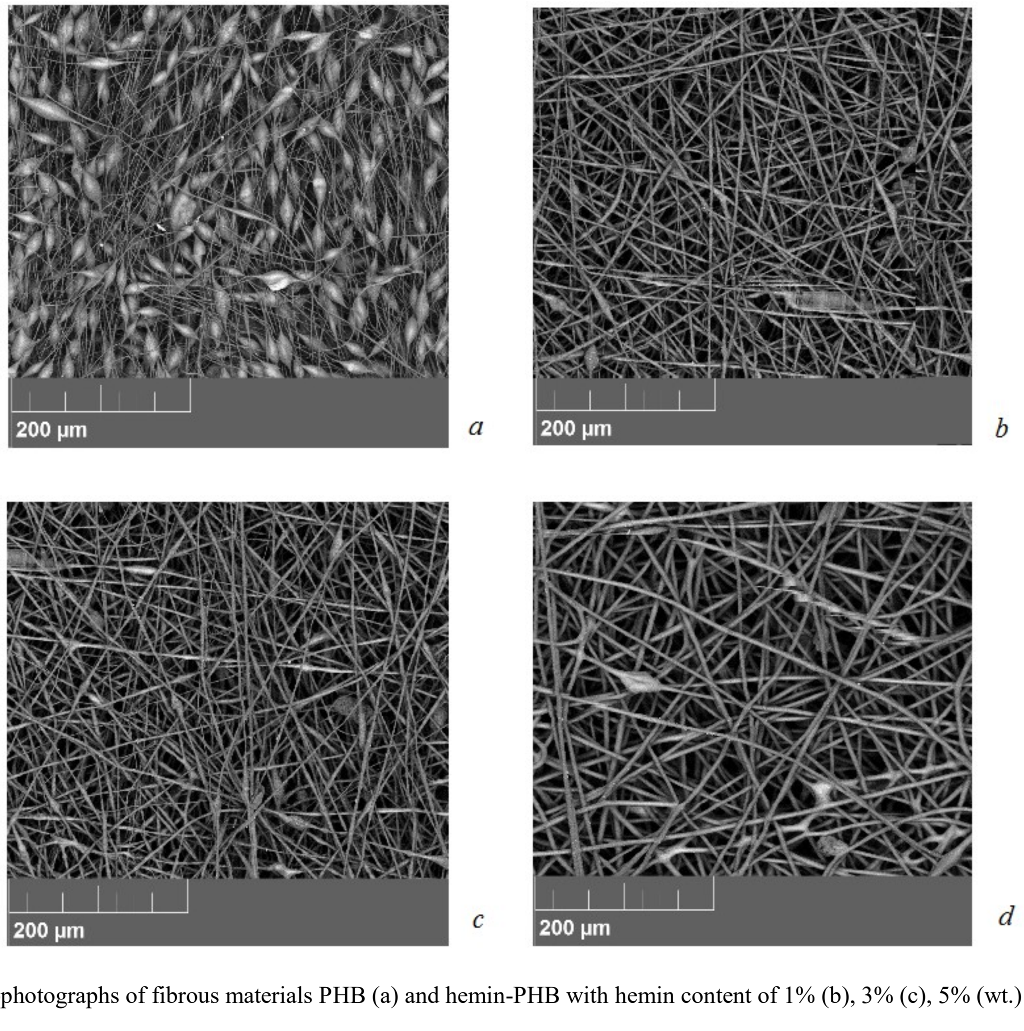
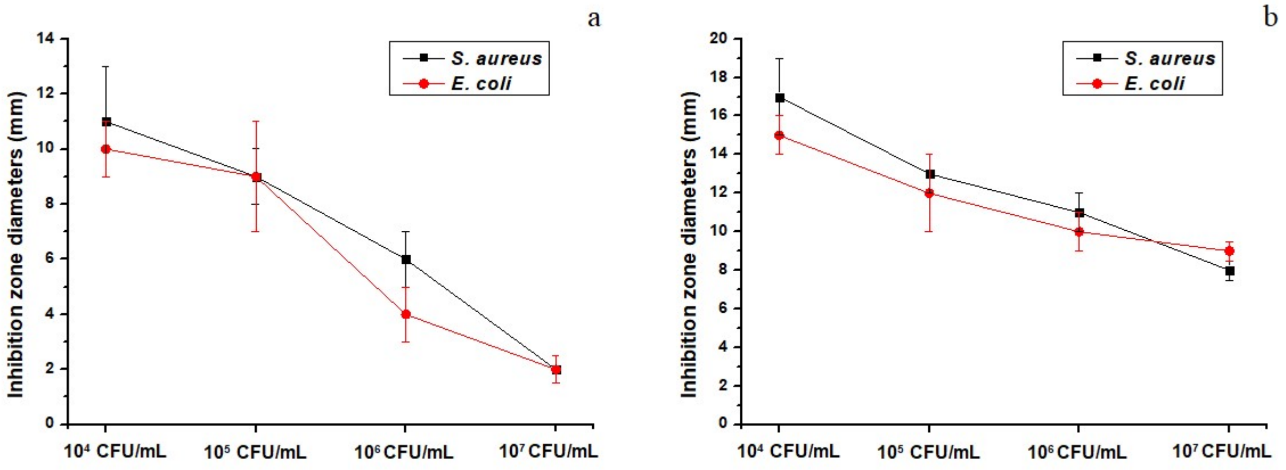


Figure 2. Microphotographs of fibrous materials PHB (a) and hemin-PHB with hemin content of 1% (b), 3% (c), 5% (wt.) (d).



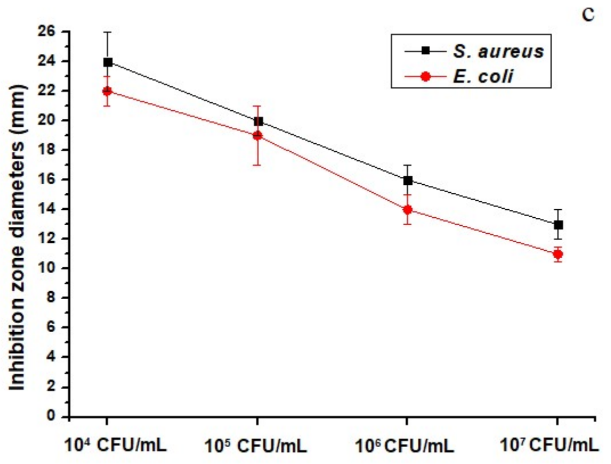


Figure 3. The inhibitory effect of hemin-PHB on *E. coli* (strain 1257) and *S. aureus* (strain 209 P). Hemin concentration (wt.%): 1% (a), 3% (b), 5% (c).

As can be seen in Figure 2, the morphology of non-woven materials is different depending on the fiber composition. With the addition of hemin metal complexes, the packing density and local orientation of the fibers in the bulk of the nonwoven material change. It can be seen that the loosest packing is observed in the case of PHB fibers and with the addition of 1% hemin, which is associated with the presence of a large number of defects. As the concentration of metal complexes increases, the compactions disappear and the fibers are stacked more densely with a predominant spatial orientation relative to neighboring fibers (Figure 2d).

Thus, the introduction of particles of a different molecular nature, such as hemin metal complexes, leads to a change in the geometric parameters of poly-hydroxybutyrate fibers, which is a consequence of the compaction and anisotropy of the nonwoven fibrous material as a whole.

The results of the inhibitory effect of hemin-PHB were taken into account by measuring the zone of growth inhibition of *E. coli* and *S. aureus* after daily incubation (Figure 3). Content of 1%, 3% and 5% hemin in the polymer matrix was 1, 3 and 5 μg/cm^2, respectively. This choice was due to the fact that, according to some literature data, metalloporphyrins in a dosage of a similar order (μg) demonstrated antimicrobial effect against microorganisms (including *S. aureus* and *E. coli*).[38,39]

It was found that 5% is the threshold content. Above this value, inclusions of hemin in the form of a separate phase of aggregated metal complex begin to predominate in the PHB matrix. In this state, hemin almost completely loses its antimicriobial effect due to the appearance of steric obstacles to interaction with oxygen and its activation. In addition, a hemin content higher than 5% begins to affect the physical and mechanical characteristics of the polymer matrix, namely, an increase in the degree of crystallinity, which makes the material more brittle.

A PHB sample without hemin (0%) was used as a control. There was no inhibition of the growth of microorganisms in the Petri dishes in which this sample was placed, which confirmed the lack of toxicity of PHB.

As can be seen from the data presented in Figure 3, the effectiveness of hemin-PHB, expressed in the degree of inhibition of the growth of *E. coli* and *S. aureus*, is directly dependent on the percentage of hemin in PHB. Thus, PHB with 5% hemin has a significant inhibitory effect on the Gram-negative and Gram-positive microorganisms used in the experiment. The diameter of the growth inhibition zone of *S. aureus* ranged from 13 to 24 mm, and that of *E. coli* from 11 to 22 mm, depending on the dilution of the bacterial suspension used for inoculation. It should be noted that *S. aureus* in this experiment showed a higher (on average, about 10%) sensitivity to hemin compared to *E. coli*.

The degree of inhibition of the growth of microorganisms by PHB with 1% and 3% hemin was expectedly lower. In dishes with hemin-PHB (3% wt.), an inhibition zone of 8-17 mm (*S. aureus*) and 9-15 mm (*E. coli*) was observed. In the case of exposure to PHB with 1% hemin, the diameter of the growth inhibition zone did not exceed 11 mm for *S. aureus* and 10 mm for *E. coli*, even at a dilution of 10^4.

Based on the literature data, it can be assumed that the inhibitory effect of hemin is associated with its peroxidase-like and monooxygenase-like activity. According to the model of antibacterial activity mechanism proposed by I. Stojiljkovic *et al.*, metalloporphyrins can enter the bacterial cell in two ways: using heme receptors or passive diffusion through the outer membrane. Next, metalloporphyrins are incorporated into the heme binding sites of cytochromes, either directly into the periplasm (or extracellular space in

Gram-positive bacteria), or after transport into the cytoplasm and export back to the periplasm using the cytochrome assembly mechanism. The incorporation of metalloporphyrins into cytochromes interrupts the transfer of electrons to oxygen, causing incomplete reduction of O_2 and the formation of reactive oxygen species (ROS).[38,39]

Conclusions

Based on the data obtained, it can be concluded that varying the hemin content in the polymer fibrous material affects the structural and functional properties. PHB fibers containing 5% hemin inhibit the growth of Gram-negative and Gram-positive bacteria *E. coli* and *S. aureus*. These microorganisms are widespread in the environment and are potential contaminants of skin damage. Thus, the resulting porphyrin-polymer system hemin-PHB can be recommended for use as an antiseptic material in the treatment of wounds.

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