

Antibacterial activity of chlorophyll polymeric form against test cultures *S. aureus* and *E. coli*

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Abstract. The increase in the number of microorganism strains with resistance to anti-bacterial and disinfecting agents is getting more and more prevalent and has become an issue when treating the human and animals diseases and carrying out measures for disinfection treatment. This problem can be solved by using photodynamic and light-independent therapy. In both areas, metalloporphyrins have been successfully used for many years. One of the most famous representatives of porphyrins is chlorophyll (Chl). This work aims to develop of Chl polymeric form by incorporation in poly(lactic acid) (PLA) and study its inhibitory effect against *Staphylococcus aureus* and *Escherichia coli*, which are known as contaminants of the mucous and skin epithelium of humans and animals. The preparation method of Chl polymeric form is presented. The degree of Chl incorporation into PLA was more than 98%. The assessment of antimicrobial activity was carried out by measuring the inhibition zone diameters after bacterial incubation for 24–96 h. It was shown that Chl and Chl-PLA at a dosage of 75 µg inhibited *S. aureus* significantly. The exclusively bacteriostatic effect on *E. coli* was observed. These results can be used in the development of dosage forms and disinfectants.

Keywords. chlorophyll, poly(lactic acid) (PLA), *Escherichia coli*, *Staphylococcus aureus*, antibacterial activity

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1 Introduction

Recently, cases of the emergence of antibiotic-resistant strains have been increasingly recorded. Therefore, both the development and implementation of new drugs with an antibacterial effect and the use of alternative approaches seem to be the urgent task. For example, photodynamic therapy (PDT) and light-independent therapy are widely used to solve this problem. In both areas, compounds based on porphyrin macrocycles, in particular metalloporphyrins, have been successfully used for many years [1–3].

One of the most famous representatives of porphyrins is the chlorophyll (Chl) – tetrapyrrole magnesium complex with two carboxyl substituents, present in all photosynthetic microorganisms. Chl was first isolated as a green pigment by J.B. Cavant and P.J. Pelletier in 1817 from plant leaves. It was subsequently discovered that Chl consists of several components. In the 1900s R. Wiltstätter purified and crystallized components *a* and *b*. Currently, components *c1*, *c2*, *d* and *f* are also known [4, 5].

Due to its unique properties, Chl has found wide application for various purposes: as a food additive (E140), a dye, in photochemical and electrochemical catalysis, as well as for the generation of singlet molecular oxygen during PDT [6–10].

The increase in the antitumor and antimicrobial activity of Chl is achieved through its inclusion in various polymeric carriers. For example, in the work of V. Rizzi et al. hydrogels based on polyamidoamines (PAA) were used to incorporate Chl isolated from the extract of seaweed *Spirulina (Arthrospira Platensis)* into the polymeric matrix [11]. In another work by the same authors, the photodynamic activity of Chl included in the biofilm consisting of chitosan and cyclodextrin mixture was studied [12]. Yu-Ra Kang et al. demonstrated the increase in Chl stability during storage by encapsulation in microcapsules from gum arabic and maltodextrin mixture [13]. For the same purpose, Zhi-Hong Zhang et al. produced microcapsules from whey protein isolate and cabbage leaf Chl [14].

Thus, as can be seen from the literature, there is a lot of data on the photodynamic activity of Chl. And at the same time, not many results have been presented on its light-independent antimicrobial action, both in free form and as part of polymeric matrix.

Therefore, the aim of this work is the developing of Chl polymeric form by incorporation in poly(lactic acid) (PLA) – non-toxicity and wide application in pharmacology and medicine as a biocompatible polymer [15, 16] and subsequent study of its inhibitory effect against test cultures of microorganisms *Staphylococcus aureus* and *Escherichia coli*, which are widely used in microbiological and biochemical studies. Also, *S. aureus* and *E. coli* are known as contaminants of the mucous and skin epithelium of humans and animals, therefore, under certain circumstances, they can cause inflammatory processes and food poisoning [17, 18].

2 Materials and Methods

The materials used in the work were chlorophyll (Chl) as Chl*a* and Chl*b* mixture (3:1), poly(lactic acid) (PLA, “Nature Works”), chloroform (CHCl₃, “Sigma-Aldrich”), dimethyl sulfoxide DMSO (C₂H₆OS, “PanReac Applichem”), 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”). A mixture of chlorophylls was extracted from dried nettle leaves (*Urtica dioica*) according to the method [19] with the additional use of cavitation mode in a rotary pulsation apparatus. The structural formulas of Chl*a*, Chl*b* and PLA are shown in Figure 1.

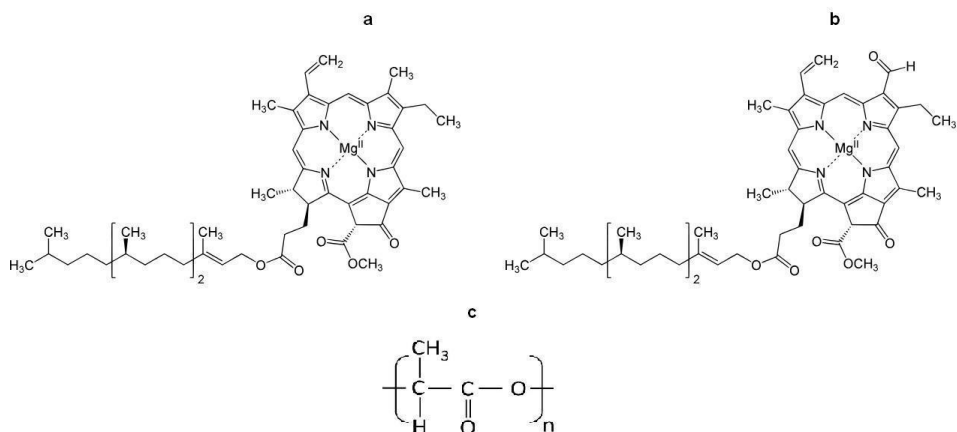


Fig. 1. Structures of Chlorophylla (a), Chlorophyllb (b) and poly(lactic acid) (c).

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 electronic absorption spectra of Chl solutions were recorded using a PE5400UF spectrophotometer (“Ekroskhim”) with a spectral range from 190 to 1000 nm. The spectra were recorded in standard quartz cells in the wavelength range $\lambda = 500\text{--}800$ nm. The absorbent layer thickness is 10 mm. Optical density scanning in a specified wavelength range, saving and loading tables of the results obtained were carried out in the SC5400 program (version 2.1). The calibration graphs and quantitative analysis were performed using QA5400 software (version 2.1). All measurements were carried out in triplicate.

To obtain the polymeric form, Chl solutions and PLA were prepared in chloroform. The PLA concentration in the chloroform solution was 5 wt.%. Chl was added to the PLA solution at a concentration of 0.1, 0.2, 0.3, 0.4 and 0.5 wt.% relative to the weight of PLA. Mixed solutions of PLA and Chl were poured onto Petri dishes and left at a temperature of 22 ± 2 °C for 48 h, then placed in a drying oven and kept at a temperature of 35 ± 2 °C for another 3 h. Dry films were separated from glass surfaces and kept in the dark until used in further work.

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 ($\lambda = 600$ 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. 10^4 CFU/mL dilution of *E. coli* and *S. aureus* was inoculated into Petri dishes with sterile MPA.

From samples of Chl-PLA fragments with an area of 1 cm² 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 °C for 24, 48, 72 and 96 h. The results were taken into account by the diameter of the growth inhibition zone (mm). The experiments were carried out in triplicate. As a comparison, Chl was used in free form with the similar dosage, which was 15, 30, 45, 60 and 75 µg, respectively. 100 µL of Chl solutions were added to wells made with a sterile piercer in MPA.

Statistical processing of the results was carried out using MS Excel software. The significance of differences in mean values was established using Student's method at a significance level of $p < 0.05$. The results were presented as the arithmetic mean and standard deviation ($M \pm m$).

3 Results and Discussion

At the initial stage of the experiment, electronic absorption spectra of Chl solutions prepared in DMSO at concentrations from 28.8 to 1.8 µg/mL were recorded. As can be seen from Figure 2a, the absorption maxima of all spectra were at the wavelength of 650 nm, which is typical for chlorophyll. To subsequently calculate the degree of Chl incorporation into the polymeric matrix, the calibration curve was constructed using QA5400 software. Figure 2b shows that the correlation coefficient (R^2) was high and amounted to 0.9999.

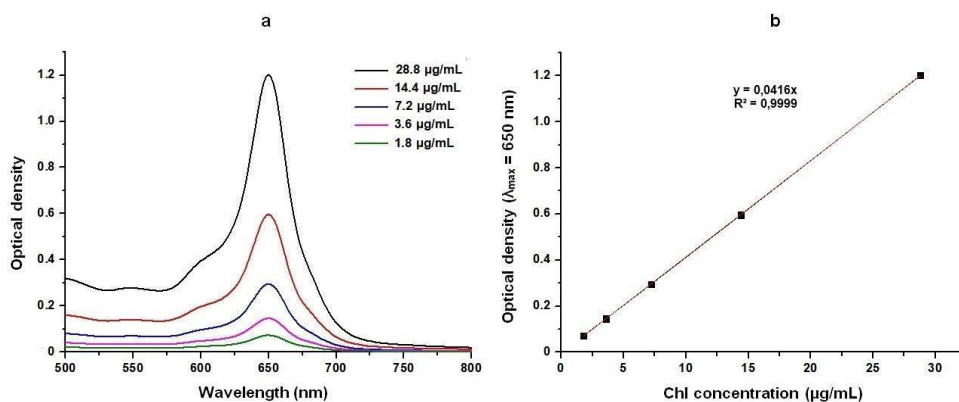


Fig. 2. Electronic absorption spectra of Chl in DMSO (a) and a dependence of optical density on Chl concentration (b).

Based on mathematical calculations, it was found that 1 cm² of polymeric film contains 15 (0.1%), 30 (0.2%), 45 (0.3%), 60 (0.4%) and 75 µg (0.5%) Chl.

To evaluate the degree of Chl incorporation into PLA, Chl was extracted using DMSO from polymeric films (1 cm²) obtained according to the method described above. Figure 3a shows the spectral profiles of extracts with 0.1–0.5% Chl content, diluted with DMSO to the same concentration of 15 µg/mL in order to avoid exceeding optical densities above 1. Then, the Chl concentration was calculated based on the optical density values and the previously obtained calibration curve. The data on Chl content in PLA, obtained both as a result of mathematical calculations and based on the calibration curve, are shown as diagrams in Figure 3b.

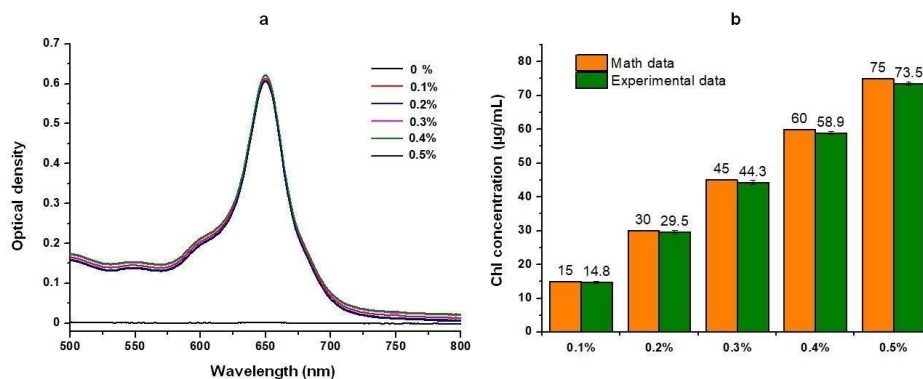


Fig. 3. Electronic absorption spectra of Chl extracts from PLA in DMSO (a), math and experimental data on Chl content in PLA ($p < 0.05$) (b).

Thus, based on the data obtained, we can conclude that the degree of Chl incorporation into the polymer was, on average, 98.3%.

The results of the inhibitory effect of Chl and Chl-PLA were taken into account by measuring the zone of growth inhibition of *S. aureus* and *E. coli* after 24, 48, 72 and 96 h incubation (Figure 4 a, b and Figure 5 a, b).

PLA sample without Chl (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 PLA.

As can be seen from the data presented in Figure 4a, in the experiment with Chl in free form, a clear correlation was observed between the content of the active substance and its effect on the growth of *S. aureus*. The diameter of inhibition zone was about 24 mm at Chl content of 15 µg/well, after 24 h; it reaches 50 mm at 75 µg/well. As time passed (observations were carried out at intervals of 24 h), the diameter of inhibition zone decreased. Moreover, this change exceeded 50% at Chl content of 15 µg/well, it was about 45% at 30 µg/well, 16% – at 45 µg/well and 9% – 60 µg/well. No decrease in the diameter of inhibition zone was observed at 75 µg/well (within the measurement error).

Thus, we can only talk about the bacteriostatic effect with 15–60 µg/well of Chl, the duration of which increases with the dosage of the drug, and with a Chl content of 75 µg/well we observe the bactericidal or long-term bacteriostatic effect of this substance, exceeding 96 h.

We observed the similar picture with Chl-PLA complex: the diameter of inhibition zone after 24 h had values comparable to the previous experiment and correlating with the Chl content (Figure 4b). However, after 96 hours and at Chl content of 15 µg/cm², the decrease in diameter of inhibition zone exceeded 57%, it was 46% at 30 µg/cm², it was less than 12% at 45 µg/cm² and 5% – at 60 µg/cm². No change in the diameter of inhibition zone was observed at Chl content of 75 µg/cm². From these data we can assume that Chl in combination with PLA has a prolonged effect compared to Chl in free form.

In experiments with the influence of Chl in free form and the Chl-PLA complex on the growth of *E. coli*, we observed the exclusively bacteriostatic effect, which was weaker and less durable than in similar experiments on *S. aureus* (Figure 5 a, b). As can be seen, the bacteriostatic effect disappeared completely after 72 h even at the maximum concentration of the active substance.

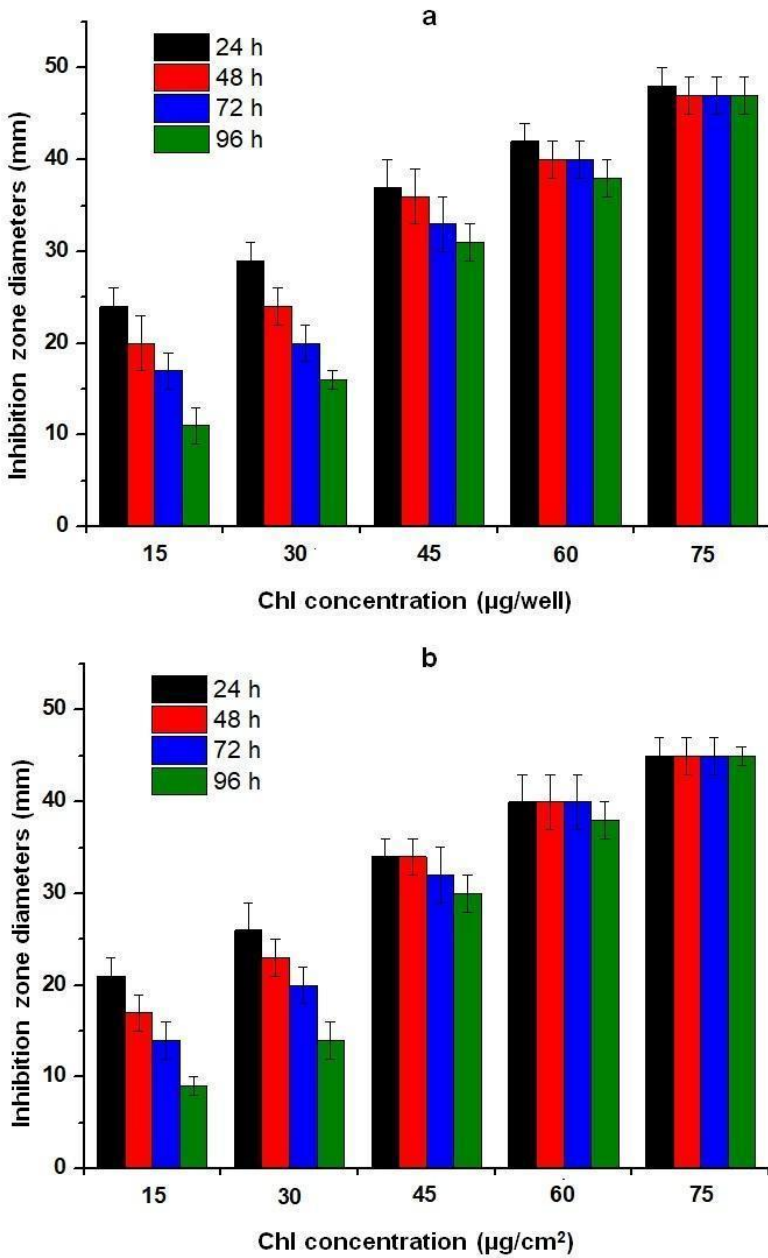


Fig. 4. The inhibitory effect of Chl (a) and Chl-PLA (b) on *S. aureus* (strain 209 P).

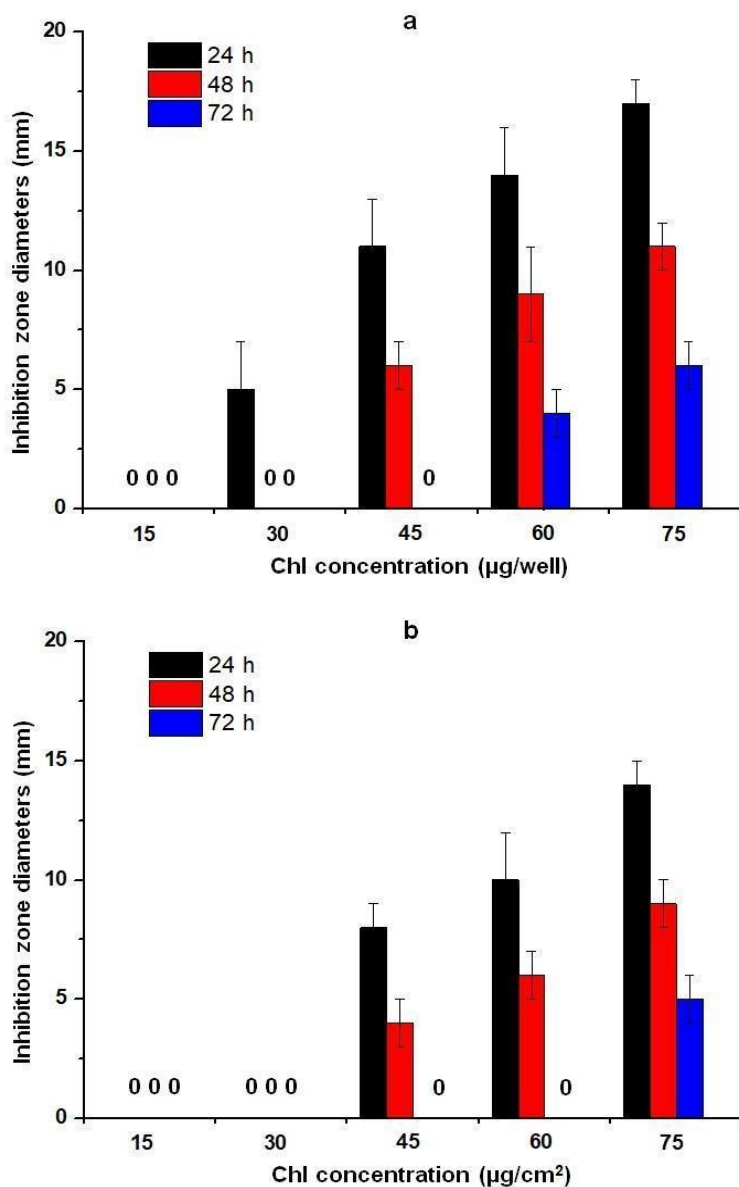


Fig. 5. The inhibitory effect of Chl (a) and Chl-PLA (b) on *E. coli* (strain 1257).

Based on the literature data, it can be assumed what the mechanism of Chl inhibitory effect is. According to the model of antibacterial activity mechanism proposed by I. Stojiljkovic, B.D. Evavold et al, metalloporphyrins can enter the bacterial cell in two ways: using haem receptors or passive diffusion through the outer membrane. Next, metalloporphyrins are incorporated into the haem 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 O₂ and the formation of reactive oxygen species (ROS) [20].

4 Conclusions

Thus, the method described in this article made it possible to obtain Chl polymeric form with the incorporation degree of more than 98%. Based on the data obtained in the microbiological test, it can be concluded that the observed antimicrobial effect of Chl and its polymeric form was directly dependent on the concentration used. It was found that the bactericidal or stable and long-term bacteriostatic effect on the growth of *S. aureus* provided Chl in free form and the Chl-PLA complex at a dosage of 75 µg. In addition, the prolonged effect of Chl-PLA was observed. These results can be used in the development of dosage forms against staphylococcal infections, as well as disinfectants.

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