

Antimicrobial properties of chlorophyll and hemin incorporated into the polymeric matrix of poly-*N*-vinylpyrrolidone

D.V. Gruznov^{1,*}, *O.A. Gruznova*², *I.P. Chesnokova*^{2,3}, *L.F. Plaksina*³, *A.V. Lobanov*^{2,4}, and *G.Sh. Shcherbakova*¹

¹All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology – Branch of Federal Scientific Center – K. I. Skryabin, Ya. R. Kovalenko All-Russian Research Institute of Experimental Veterinary Medicine, Russian Academy of Sciences, 123022, Moscow, Russian Federation

²Semenov Federal Research Center for Chemical Physics, Russian Academy of Sciences, 119991, Moscow, Russian Federation

³MIREA – Russian Technological University, 119454, Moscow, Russian Federation

⁴Moscow Pedagogical State University, 119991, Moscow, Russian Federation

Abstract. The increase in the number of antibiotic-resistant strains of microorganisms is becoming more widespread. Metalloporphyrins are promising and modern antimicrobial agents. The most well-known representatives of metalloporphyrins are chlorophyll (Chl) and hemin. This paper presents the results of studies on the effectiveness of Chl and hemin complexes with poly-*N*-vinylpyrrolidone (PVP) as an antimicrobial agent against *Staphylococcus aureus* and *Escherichia coli*. The method for preparing polymeric forms of Chl and hemin is presented. The binding constants of these substances to the polymer were calculated, which were 0.5×10^5 L/mol for Chl and 3.3×10^4 L/mol for hemin. Experimental data on the release of substances from the polymeric matrix were obtained. It was found that the complete release of Chl from PVP was observed after 13 h, and hemin – after 10 h. The data on the comparative antimicrobial effect of substances in free and polymeric form were obtained in a microbiological test. Further these results can be used in the development of medicines against microbial infections.

1 Introduction

In recent years, the increasing number of microorganisms resistant to antibiotics and disinfectants poses a serious and global problem [1, 2]. Thus, the urgent task is not only the search and implementation of new drugs, but also the use of such approaches as the application of photodynamic (PDT) and light-independent therapy. Metalloporphyrins have been widely used in both fields for many years [3]. Some of the most well-known representatives of porphyrins are chlorophyll (Chl) and hemin [4, 5].

The basic skeleton of Chl is a large, flat structure with a symmetrical arrangement, in which four pyrrole rings are connected by methine (–CH=) bridges, and four nitrogen

* Corresponding author: 79164422245@yandex.ru

atoms are coordinated to a central metal atom (magnesium). In addition, Chl molecules have a phytol group, which imparts hydrophobic properties and the ability to integrate into the lipid layer of biological membranes [6].

The first information about Chl dates back to 1817, when two French chemists Peltier and Quantou published the work entitled “Notes on the Green Matter of Leaves”. Due to its special chemical, photochemical and photophysical properties, Chl has found wide application in the photoconversion of solar energy, in semiconductor technology, in photochemical and electrochemical catalysis, for the generation of singlet molecular oxygen, for thermal and photostabilization of fats and polymers, and also as a dye in textiles, paint and varnish, food and other industries [7].

Hemin is an organic compound, one of the most famous representatives of natural porphyrins, containing the iron cation Fe^{3+} and the coordinating chloride anion Cl^- [8].

Hemin was first obtained by crystallization from the blood of L.K. Teichmann in 1853. Hemin is widely used in pharmacology to create drugs that correct heme deficiency in the body – “Normosang”, “Pangematin”, and is also used in antitumor therapy [9, 10].

To increase the effectiveness of porphyrins, various approaches are used, in particular, their incorporation into the matrix of a polymeric carrier. It should be noted that in recent years, special preference has been given to biocompatible and biodegradable polymers with minimal toxic effects. Poly-*N*-vinylpyrrolidone (PVP) has all of the above properties [11].

This paper presents the results of assessing the antibacterial activity of Chl and hemin included in the PVP polymeric matrix against gram-negative (*Escherichia coli*) and gram-positive (*Staphylococcus aureus*) microorganisms. These bacteria are one of the most common causes of antibiotic-resistant infections, as well as contaminants of the mucous and skin epithelium of humans and animals, capable of causing inflammatory processes and food poisoning [12].

2 Material and methods

The materials used in this work were chlorophyll (Chl) as Chla and Chlb mixture (3:1), hemin (porcine, 99.5%, “BioFroxx”), poly-*N*-vinylpyrrolidone (PVP, $M_w = 10000$ g/mol, “Ataman Kimya”), dimethyl sulfoxide DMSO ($\text{C}_2\text{H}_6\text{OS}$, “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”). Chl was extracted from dried nettle leaves (*Urtica dioica*) [13].

The structures of Chla, Chlb, hemin and PVP are shown in Figure 1.

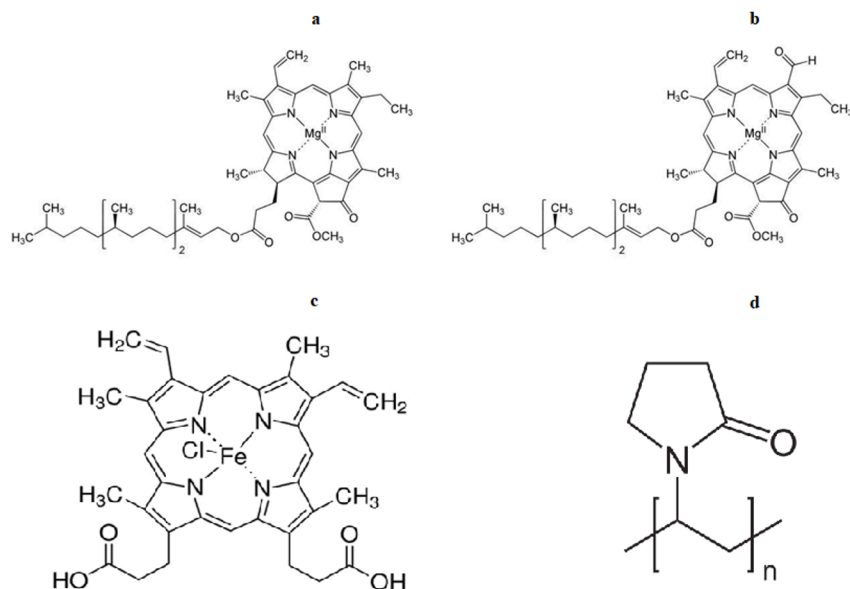


Fig. 1. Structures of Chla (a), Chlb (b), hemin (c) and poly-*N*-vinylpyrrolidone (d).

2.1 Recording of electronic absorption spectra

The electronic absorption spectra of Chl and hemin solutions were recorded using a PE5400UF spectrophotometer (“Ekroskhim”) with a spectral range from 190 to 1000 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 software (version 2.1).

2.2 Preparing of Chl polymeric forms

To obtain the polymeric form, PVP was dissolved in water to a concentration of 5% (wt.%). In parallel, solutions of Chl was prepared in DMSO at concentrations of 0.1, 0.2, 0.3, 0.4 and 0.5 wt.% relative to the weight of PVP. The resulting solutions of polymer and substances were mixed and stored at room temperature in the dark until experiments were carried out.

2.3 Preparing of hemin polymeric forms

To obtain the polymeric form, PVP was dissolved in water to a concentration of 6% (wt.%). In parallel, solutions of hemin were prepared in DMSO at concentrations from 40 to 320 $\mu\text{g/mL}$. Then the resulting solutions of polymer and substances were mixed. Thus, the final concentration of hemin in PVP ranged from 20 to 160 $\mu\text{g/mL}$, and PVP – 3% (wt.%).

2.4 Binding constant calculation

The binding constant (K_d) of porphyrins to PVP was calculated using mass concentrations [14]. For this purpose, the mole fraction (Θ) of Chl or hemin was calculated using formula 1 ($\lambda_{\text{max}} = 650$ and 403 nm, respectively):

$$\Theta = (A - A_o) / (A_{\infty} - A_o), \quad (1)$$

A_o is the optical density at a mass concentration of PVP equal to 0%, and A_{∞} is the optical density at its maximum mass concentration.

Next, to construct a scatter plot along the Y -axis, Θ was subtracted from 1 ($1-\Theta$). The molar concentration of the polymer $[P]$ was calculated using formula:

$$[P] = (m(\text{PVP}) / M_w) / V, \quad (2)$$

Then, to construct a scatter plot, the value $-\Theta/[P]$ was calculated along the X -axis. After calculating all parameters in MS Excel, graphs were drawn, trend lines were drawn, and $\text{tg}\alpha$ was calculated. The binding constant (K_d) was calculated using the following formula:

$$K_d = 1/\text{tg}\alpha, \quad (3)$$

2.5 Studying kinetics of Chl and hemin release from polymeric matrix

To study kinetics of Chl or hemin release from the polymeric matrix *in vitro*, 6 mL of solution of the polymeric form of Chl or hemin (with Chl and hemin concentration of 14.4 and 2 $\mu\text{g/mL}$, respectively) was placed in a dialysis tube (polypropylene with $M_w = 7000$ g/mol, "School Supplies Store") and dialysis against phosphate-buffered saline (PBS) with pH 7.2-7.4 at 37 °C. Then, every 20-40 min, a polymeric form was sampled from the dialysis bag to determine the concentration of Chl or hemin. For these purposes, a PE5400UF spectrophotometer (Ecrochem) and QA5400 software (version 2.1) were used.

2.6 Microbiological test

To obtain daily cultures of *E. coli* (strain 1257) and *S. aureus* (strain 209-P), they were reseeded and further cultivated on a slanted MPA in a thermostat (37 °C, air-dry 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 suspended matter were confirmed spectrophotometrically ($\lambda = 600$ nm). Next, successive dilutions were prepared from suspensions of daily cultures of *E. coli* and *S. aureus* (10^9 CFU/mL) in 10-fold steps: 10^8 CFU/mL, 10^7 CFU/mL, 10^6 CFU/mL, 10^5 CFU/mL and 10^4 CFU/mL by titration in sterile saline solution. The dilution at a concentration of 10^4 CFU/mL was inoculated into Petri dishes with sterile MPA. 100 μL of Chl or hemin in complex with PVP was added to wells made with a sterile punch in the center of the MPA. Then incubation was carried out in thermostat at temperature of 37 °C for 120 h, taking into account the daily results for the diameter of the growth inhibition zone (mm).

3 Results and discussion

At the initial stage, electronic absorption spectra of Chl and hemin solutions prepared in DMSO were recorded at concentrations from 1.8 to 28.8 $\mu\text{g/mL}$ and from 0.25 to 4 $\mu\text{g/mL}$, respectively. As can be seen from Figure 2, the absorption maxima of the spectra were at wavelengths of 650 and 403 nm, which is typical for these compounds.

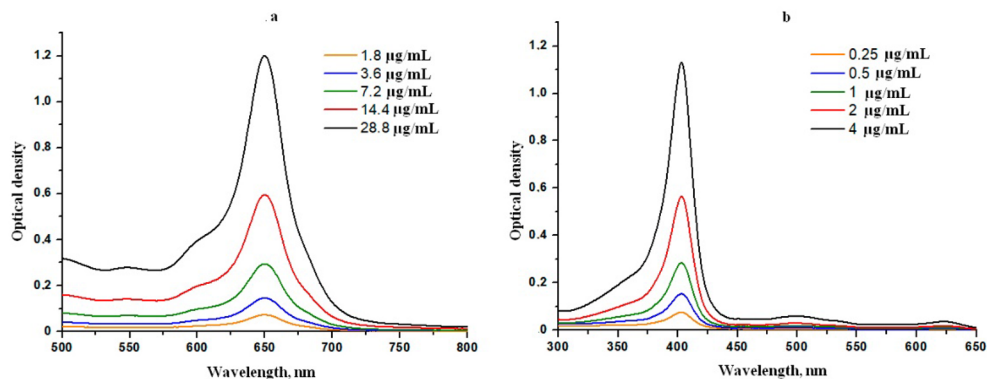


Fig. 2. Electronic absorption spectra of Chl (a) and hemin (b) in DMSO.

Next, the binding constant of these compounds to PVP was calculated. Figure 3 shows the electronic absorption spectra of polymer complexes of Chl with PVP in concentrations from 0 to 5% (wt.%) and hemin with PVP in concentrations from 0 to 3% (wt.%), as well as dot plots with drawn trend lines, constructed from the data obtained.

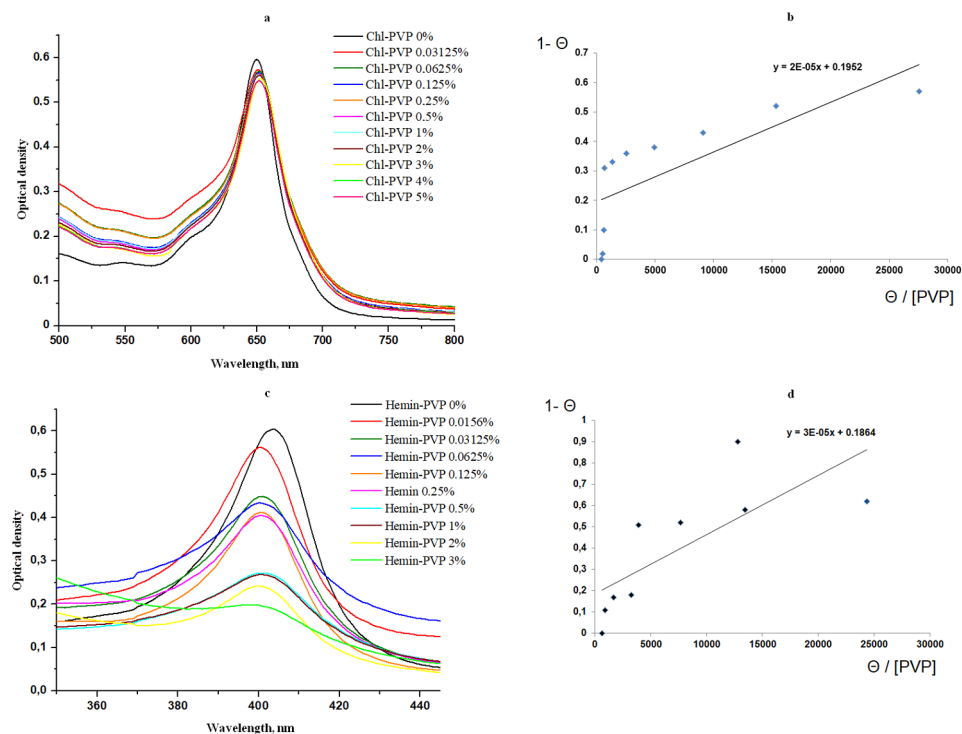


Fig. 3. Electronic absorption spectra of the Chl-PVP complex (a) and hemin-PVP (c), dot plots with trend lines (b, d).

Based on the results obtained, the binding constants were calculated to be 0.5×10^5 and 3.3×10^4 L/mol for the Chl-PVP and hemin-PVP, respectively. Also, it should be noted that from the data in Figures 3a and 3c it is clear that the absorption bands of Chl and hemin are broadened, which may be due to the manifestation of coordination and other non-covalent

interactions with polymer macromolecules and, possibly, with the local concentration of porphyrin molecules in areas of binding with PVP macromolecules.

At the next stage, the kinetics of Chl and hemin release from the PVP polymeric matrix was studied (Figure 4).

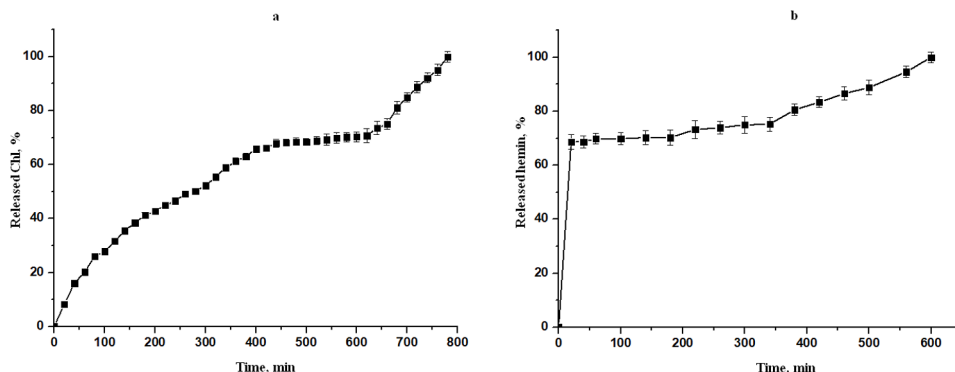


Fig. 4. Chl (a) and hemin (b) release profile from the PVP polymeric matrix at pH 7.4 and 37 °C.

As can be seen from the data presented, a rather gradual release of Chl from PVP was observed. 99.95% of the substance was released in 780 min (13 h). In the first 20 min, a rapid release of hemin from PVP was detected (about 70%). Then a stage of its slower exit was noted. As a result, 99.98% of hemin was released from the polymer in 600 min (10 h).

The results of antibacterial activity were recorded daily by measuring the inhibition zone diameters (Fig. 5, Fig. 6). In the diagrams (Fig. 5) we can see that with comparable diameter of the growth inhibition zone of microorganisms at the beginning of the experiment, their decrease occurs more slowly when using the Chl-PVP complex. From this we can conclude that this complex has a prolonged effect compared to free substance.

Based on the results of the study of the antimicrobial properties of hemin, shown in the diagrams (Fig. 6), we can conclude that hemin in combination with the polymer has higher activity and duration of action against *S. aureus* and *E. coli*. This is best seen with a dose of hemin of 160 µg: the intensity of the antimicrobial effect of the polymer complex on *Staphylococcus aureus* exceeds that of solution in DMSO by 25-30%. In experiments with *Escherichia coli*, such a significant increase in antimicrobial activity was not observed.

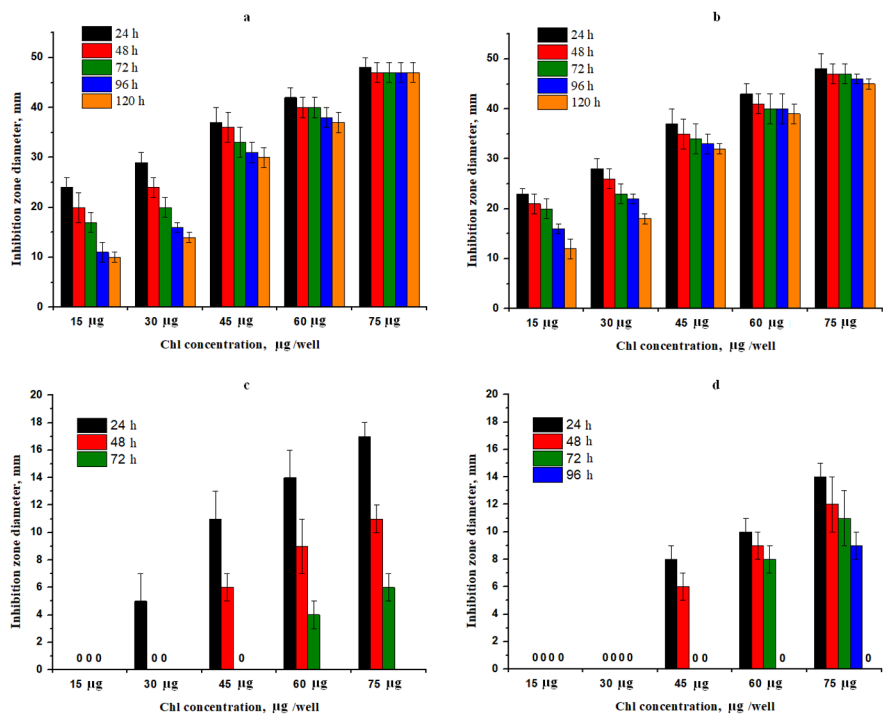


Fig. 5. Inhibitory effect of Chl (a, c) and Chl-PVP (b, d) against *S. aureus* (a, b) and *E. coli* (c, d).

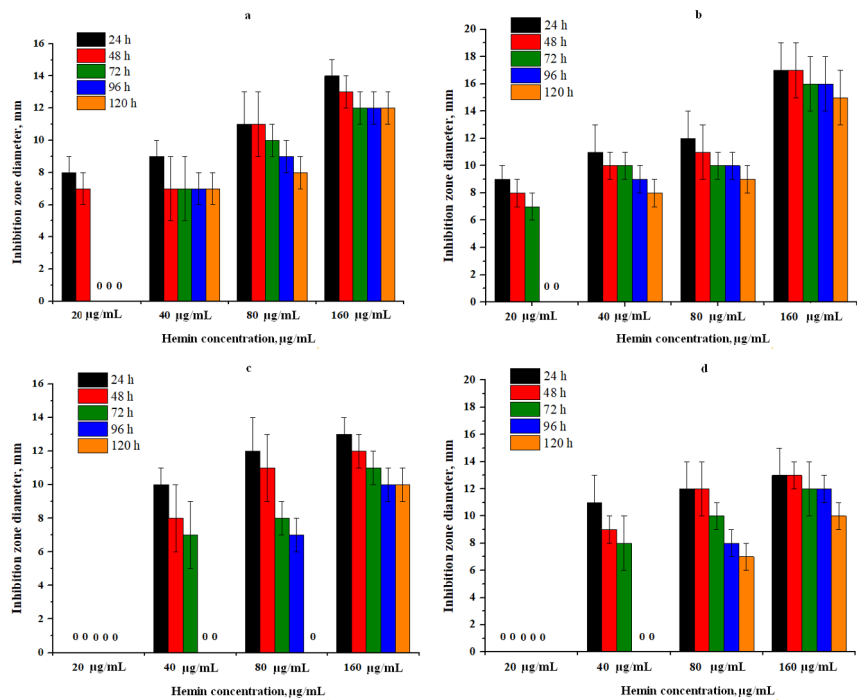


Fig. 6. Inhibitory effect of hemin (a, c) and hemin-PVP (b, d) against *S. aureus* (a, b) and *E. coli* (c, d).

4 Conclusions

Thus, based on the data obtained, the following conclusions can be drawn. Chl in free form and the Chl-PVP complex at a dosage of 15 µg have a bacteriostatic effect on the growth of *S. aureus*, and from 45 µg on the growth of *E. coli*. Moreover, the antimicrobial effect of Chl and its polymeric form is directly dependent on the concentration of the active substance. The polymeric complex hemin-PVP has the prospect of practical use as an alternative antimicrobial agent with a prolonged effect against gram-positive and gram-negative microorganisms.

Further these results can be used in the development of medicines against microbial infections.

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