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Antioxidant activity of polymeric biocide polyhexamethylene guanidine hydrochloride

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Cationic polymer polyhexamethylene guanidine hydrochloride (PHMG-Cl) is promising biocide that combines a broad spectrum of antimicrobial activity, moderate toxicity, as well as reasonable cost. It is widely used as an effective disinfectant in cooling systems, swimming pools, and hospitals, personal hygiene products, etc. Recently PHMG-Cl was found to have pronounced anti-inflammatory and wound healing properties and therefore may be used for the treatment of chronic wounds and thermal burns. This may indicate the antioxidant activity of polymeric biocide.

In this study, PHMG-Cl has been synthesized by melt polycondensation of guanidine hydrochloride and 1,6-hexamethylenediamine. The structure of the cationic polymer was confirmed by ^{1}H NMR and IR spectroscopy. The viscosity-average molecular weight of PHMG-Cl was found to be 10700. The antioxidant activity of PHMG-Cl has been studied by using different methods. In the methylene blue (MB) dye test, the oxidation of MB by hydroxyl radicals generating in Fenton's system was found to decrease in the presence of PHMG-Cl in a molar ratio to MB of 5:1 and 10:1 (by 26 % and 38 %, respectively). At the same time, complete dye oxidation was observed when guanidine hydrochloride was used instead of PHMG-Cl.

The antioxidant activity of PHMG-Cl has also been studied in the model system of radical chain oxidation of benzyl alcohol (BA). In this system, alkyl and peroxyl radicals are formed. The antioxidant activity was determined by a decrease of the initial rate of oxygen absorption during the initiated oxidation of BA. The introduction of PHMG-Cl into the oxidized system in the concentrations ranged from $1.3\cdot10^{-3}$ - $1.6\cdot10^{-2}$ mol/l decreased the oxidation rate of BA by 4.5–88 %. This result demonstrates that PHMG-Cl effectively inhibits radical chain oxidation of BA. However, further research is needed to elucidate the mechanism of free radical deactivation by a polymer biocide.

Keywords: polyhexamethylene guanidine, polymeric biocide, antioxidant activity, methylene blue, radical chain oxidation, oxygen absorption

Introduction

Polymeric biocides comprising guanidinium cations in polymer backbone such as polyhexamethylene biguanide hydrochloride (PHMB-Cl) and polyhexamethylene guanidine hydrochloride (PHMG-Cl) are being considered as valuable and cheap alternatives to common antimicrobial agents because of the combination of high efficacy in killing antibiotic-resistant bacteria and fungi, as well as low toxicity to human cells [1-5]. The said cationic polymers are widely used as effective disinfectants in cooling systems, swimming pools and hospitals, in personal hygiene products, as well as in the food industry [2-4]. The high activity of guanidinium-based polymeric biocides against microorganisms is caused by the presence of multiple positive charges within a single molecule that are able to compensate the negative charges present on the outer cell membranes of microbes. Therefore, cationic polymers attack the cellular envelope, and subsequently associates itself with the head groups of the acidic phospholipids, destabilizes the osmotic equilibrium, and destructs the cytoplasmic membrane, causing leakage of a cell. The presence of hydrophobic aliphatic chains in the backbone ensures a better partition of polymeric biocide to the hydrophobic regions of the phospholipids membrane, resulting in a change of membrane permeability and lethal leakage of cytoplasmic materials [6-8].

From a practical point of view, PHMG-Cl has certain advantages over PHMB-Cl. Thus, it is much cheaper than PHMB-Cl due to simple one-step synthesis [9]. Moreover,

the acute toxicity studies on rats have shown that the median lethal dose (LD_{50}) for PHMG-Cl is 600 mg/kg [10] whereas LD_{50} value for PHMB-Cl is 25.6 mg/kg [11]. Caballero Gómez and co-authors [12] tested PHMG-Cl against a cocktail of six *Staphylococcus aureus* strains (including three methicillin-resistant strains) in the planktonic state, as well as in biofilms. Their studies revealed antibiofilm activity of polymeric biocide that was significantly higher compared to common antimicrobial agents benzalkonium chloride, cetrimide, hexadecylpyridinium chloride, and chlorhexidine [12].

Doroshenko and co-authors [13] studied the effect of a nanosized silica composite with PHMG-Cl on the immunological parameters and oxidative-antioxidant homeostasis in the blood and in the lesion of rats with an uninfected thermal burn. The composite effectively reduced the level of proinflammatory and increased the level of pro-inflammatory cytokines, indicating the presence of anti-inflammatory properties. Moreover, it also normalized the oxidative-antioxidant homeostasis by normalizing the content of markers of free radical oxidation and oxidation to accelerate the treatment of thermal burns [13]. The results of subsequent medico-biological studies confirmed the antioxidant activity of guanidine-containing polymers. For instance, the wound-healing effect of a hydrogel based on PHMG-Cl under conditions of thermal shock modeling has been studied [14]. The polymeric biocide was found to activate the growth of antioxidants and leukocytes in the blood of animals, which indicates a pronounced reparative effect.

Overall, the results of recent studies have shown that PHMG-Cl is an extremely promising biocide of complex action which combines a broad spectrum of antimicrobial activity, pronounced anti-inflammatory and wound healing properties and therefore may be used for the treatment of chronic wounds and thermal burns. In chronic wounds, there are numerous sources of reactive oxygen species including hydrogen peroxide, superoxide anions, and hydroxyl radicals, which are able to cause severe damage to cellular components [15, 16]. Patients with chronic wounds have also been shown to have low levels of the antioxidant molecules that are important for detoxifying. However, the direct activity of PHMG-Cl against free radicals has not yet been studied.

The aim of this article was to study the antioxidant activity of polymeric biocide PHMG-Cl by using different methods.

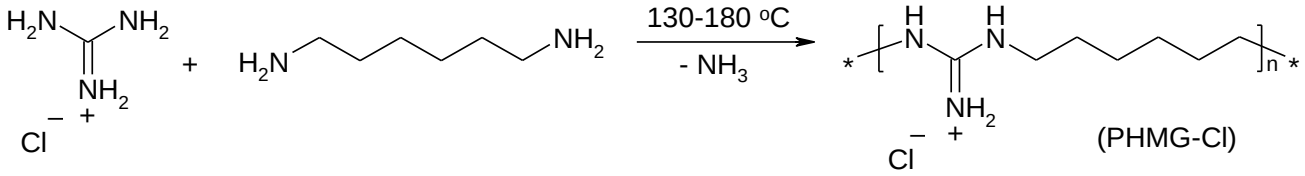
Materials and methods

Following chemicals were used in this research: guanidine hydrochloride (98 %), hexamethylenediamine (98 %), methylene blue hydrate (95 %), iron (III) acetylacetonate (97 %), benzyl alcohol (98 %) (Sigma-Aldrich), 2,2'-azo-*bis*-isobutyronitrile (99 %) (Vesta Chemicals), hydrogen peroxide (35 %), iron (II) sulfate heptahydrate (99 %), (Synbias, Ukraine).

PHMG-Cl was synthesized by melt polycondensation of guanidine hydrochloride and hexamethylenediamine [17], according to the Scheme 1.

The mixture of guanidine hydrochloride (10 g, 0.1 mol) and hexamethylenediamine (11 g, 0.095 mol) was put into a round-bottomed flask (250 ml) equipped with a mechanical stirrer. At first, it was heated to 80 °C and the melt was stirred for 2 h. Further, the reaction was carried out for 4 h at 120-130 °C, 4 h at 160 °C, and 3 h at 180 °C to obtain viscous melt. The vitreous solid of PHMG-Cl was obtained after cooling to room temperature. It was dissolved in water (100 ml), filtered, and precipitated by the addition of a saturated water solution of sodium chloride (50 ml). The polymer was isolated by decantation of water solution, dried at 130 °C for 24 h, and powdered in a porcelain mortar. It has a melting point of 136 °C.

^{1}H NMR technique was used to characterize the structure of PHMG-Cl. The spectrum was recorded with a Varian Gemini-2000 (400 MHz) NMR spectrometer in the DMSO-d6 solution. Chemical shifts were reported downfield in parts per million (ppm, δ) from a tetramethylsilane reference. The vibrational properties of polymeric biocide were studied using a Bruker Tensor-37 Fourier Transform Infrared spectrometer (Germany). The sample was prepared in KBr pellets. The spectrum was collected over the range of 400–4000 cm^{-1} at a resolution of 4 cm^{-1} in a dry atmosphere.



Scheme 1. **Synthesis of polymeric biocide PHMG-Cl**

The intrinsic viscosity of PHMG-Cl was measured with a Ubellode viscometer at 25 °C. Polymer solutions in 0.1 N NaCl was were used to suppress the polyelectrolyte effect. The polymer stock solution at a concentration of 5.0 g/100

ml was serially diluted to concentrations of 4.0, 3.33, 2.5, 2.0, 1.66 g/100 ml. At these concentrations, the relative viscosity was in the range 1.35–1.11. The values of the reduced viscosity (η_{red}) were calculated as a ratio of specific viscosity to concentration (η_{sp}/c). The intrinsic viscosity was determined by extrapolating these values to zero concentration.

The scavenging activity of PHMG-Cl against hydroxyl radicals was evaluated using the qualitative methylene blue (MB) dye test described in [18, 19]. A Fenton system was used as the source of hydroxyl radicals which are produced by the oxidation of ferrous ions and the reduction of hydrogen peroxide.

In the MB dye test, the hydroxyl radical reacts with MB cation (which is dark blue) to produce a hydroxide ion and a MB radical cation (which is colorless) [19]. The stock solutions of MB ($C=3\cdot10^{-4}$ mol/l), ferric (II) sulfate ($C = 3.5\cdot10^{-3}$ mol/l), hydrogen peroxide ($C = 4.5\cdot10^{-2}$ mol/l), guanidine hydrochloride ($C=3\cdot10^{-3}$ mol/l) and PHMG-Cl ($C=3\cdot10^{-3}$ mol/l) were prepared. For control experiments, the reactionary mixture was prepared by mixing of MB stock solution (1 ml) with certain volumes of $FeSO_4$ and H_2O_2 solutions, and the total volume was adjusted to 10 ml with deionized water. Thus, the concentration of MB in such mixtures was $3.5\cdot10^{-5}$ mol/l, whereas $FeSO_4$ to MB molar ratio was in the range from 1:1 to 10:1. The molar ratio of H_2O_2 to $FeSO_4$ was 15:1 in all cases. For testing antioxidant activity, the molar ratio of $FeSO_4$ to MB in the reactionary mixture was constant (5:1 or 10:1), whereas PHMG-Cl to MB molar ratio was in the range from 1:1 to 30:1. Guanidine hydrochloride was used as a reference compound instead of PHMG-Cl. The pH value was adjusted to the value of 3-4 by addition of diluted sulfuric acid.

UV-visible spectra were recorded over a wavelength range from 190 to 800 nm by using a Jenway 6850 spectrometer (Great Britain). The calibrating graph was obtained by measuring the absorbance of methylene blue aqueous solutions in the concentration range from $1\cdot10^{-6}$ - $1\cdot10^{-5}$ mol/l at 664 nm [18].

The antioxidant activity of PHMG-Cl was studied in the model reaction of radical chain oxidation of benzyl alcohol (BA) at 50 ºC and constant rate of free radicals initiation ($W_i = 2.98\cdot10^{-8}$ mol/l·s) [20]. 2,2'-azo-*bis*-isobutyronitrile was used as a free radical initiator. It was purified by recrystallization from ethanol and dried in a vacuum at 30 ºC. BA was purified from possible inhibiting impurities by one-off passing through a column with absorbent aluminum oxide and carbon followed by vacuum distillation (3-5 kPa) in argon atmosphere in the presence of iron (III) acetylacetonate. The volume of oxygen absorbed by the reactionary system was determined volumetrically. Antioxidant activity was determined by the decrease in the initial rate of oxygen absorption during the initiated oxidation of BA. Under oxidative kinetic conditions the gas meter allows to determine the rate of oxygen absorption W in the range 10^{-8} -10^{-4} mol/(l·s) at a substrate conversion of 0.1–0.3 mol/l. The research error was 3–6 %.

Results and discussion

The molecular weight of PHMC-Cl was calculated using the Mark-Houwink equation: $[\eta] = K\cdot M^{\alpha}$, where $[\eta]$ is intrinsic viscosity, M is the viscosity-average molecular weight, α and K are parameters, whose values depend on the nature of the polymer and solvent. For PHMG-Cl-water system, $K = 1.83\cdot10^{-3}$, $\alpha = 0.38$ at 25 °C [21]. The determined intrinsic viscosity was 0.062. Thus, the molecular weight of the synthesized polymeric biocide is 10700.

Figure 1 contains ^{1}H NMR spectrum of PHMG-Cl. The sharp peaks at 1.31 and 1.46 ppm are the signals of methylene protons a and b, respectively. The sharp and splitting peak at 3.16 ppm is assigned to protons c of NCH_2 group [6]. The broadening and splitting peaks at 7.52 and 7.81 ppm are assigned to the resonance of guanidine protons C-NH-C and $C=NH_2$+ [9].

The IR spectrum of PHMG-Cl (Fig. 2) presents a major band between 1528 and 1697 cm^{-1}, assigned mainly to the stretching vibrations of C=N groups, broad bands with maxima at approximately 3160 and 3270 cm^{-1} corresponding to the N-H stretching vibrations and the two well-defined peaks at 2930 and 2855 cm^{-1} attributed directly to the asymmetric and symmetric stretching vibrations of the methylene groups, respectively. The band at 1470 cm^{-1} is assigned to the bending vibrations of CH_2 groups [9, 21].

The MB dye test qualitatively indicates the presence of hydroxyl radicals which are formed during the Fenton's reaction through distinct bleaching of the MB dye in the tested solution [18, 19]. Baseline experiments were performed to determine the conditions ($FeSO_4$/MB ratio) at which rapid and deep degradation of the dye occurs.

Figure 3 contains UV-Vis absorption spectra of MB solutions after 10 min treatment with Fenton system at different Fe^{2+} concentrations. Significant decrease of the 664 nm peak intensity (by 75 %) was observed at $FeSO_4$ to MB molar ratio of 5:1 (violet line). The ten-fold excess of ferric ions led to complete dye oxidation (blue line). Figure 4 demonstrates the inhibiting effect of PHMG-Cl on MB degradation in Fenton system at Fe^{2+}/MB molar ratio of 5:1. The presence of PHMG-Cl in equimolar ratio to the dye did not prevent its complete oxidation after 20 min (yellow line). However, at five-fold molar excess of PHMG-Cl over MB, only 74 % of the dye was degraded during the same time (pink line). Further increase of PHMG-Cl/MB molar ratio to 10:1 significantly inhibited dye degradation. Thus, 38 % of MB remained in the solution (Fig. 4, blue line). At a higher Fe^{2+}/MB molar ratio of 10:1 in Fenton system, 25 % of MB was detected in solution after 20 min (Fig. 5, green line).

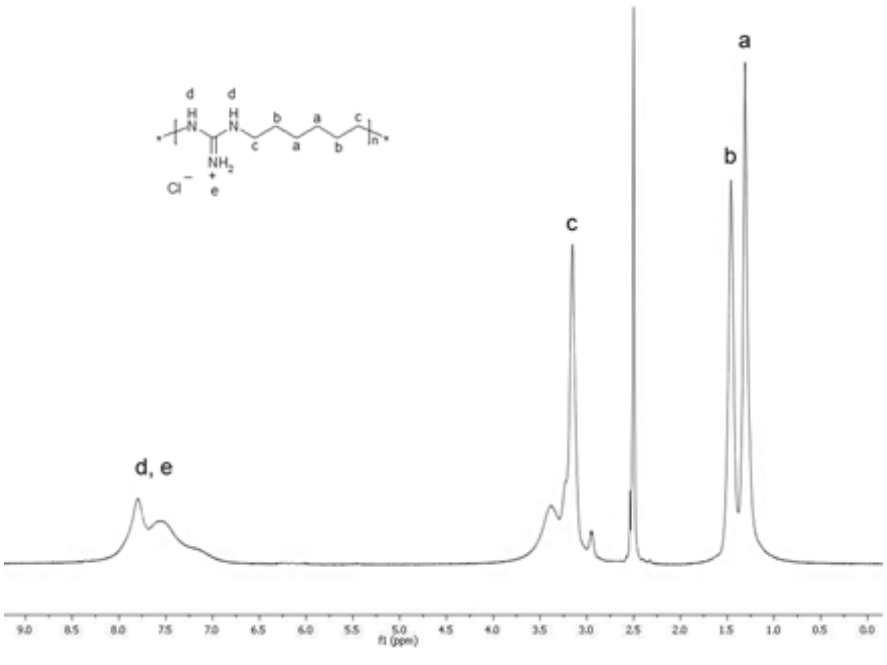


Figure 1. ^{1}H NMR spectrum of PHMG-Cl

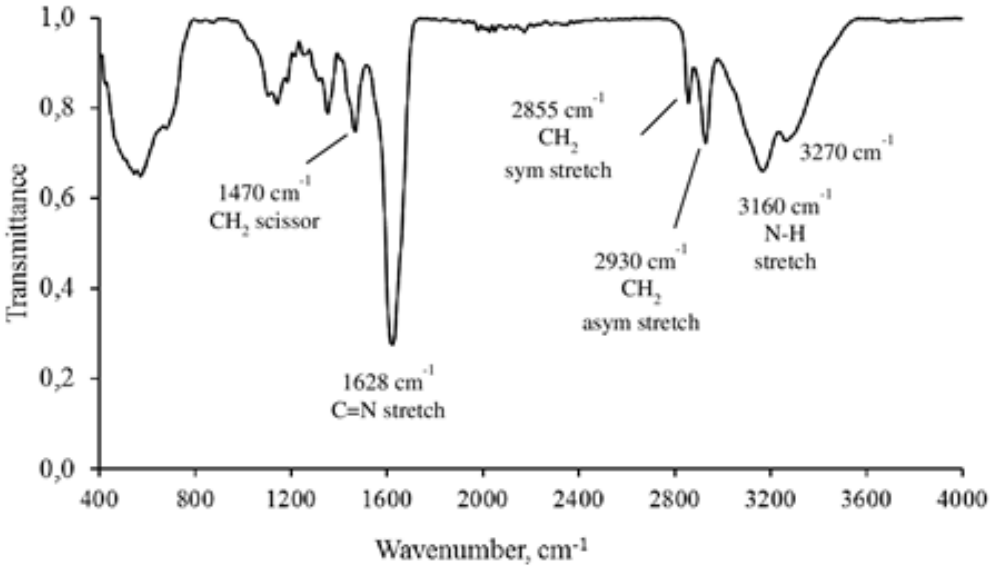


Figure 2. IR-spectrum of PHMG-Cl

Overall, the obtained results demonstrate the capacity of PHMG-Cl to deactivate hydroxyl radicals. However, guanidine hydrochloride was reported to be inert toward these highly reactive particles [22, 23]. On the other hand, 1-methylguanidine, 1-ethylguanidine, and 1,1-dimethylguanidine were found to possess direct scavenging activity against hydroxyl radicals with the formation of carbon-centered radicals [22, 23]. The authors reasonably assumed that the difference in the reactivity of hydroxyl radicals toward guanidine derivatives might be attributed to the ease of H-abstraction from their alkyl groups. The results of our study confirm this assumption. Thus, even ten-fold molar excess of guanidine hydrochloride over MB did not prevent complete dye discoloration in experimental conditions (Fig. 5, black line; Fig. 6, b 2).

In the model system of initiated oxidation of BA ($PhCH_2OH$) two types of free radicals are formed: alkyl $R^{\cdot}$ ($PhCH^{\cdot}OH$) and peroxyl $ROO^{\cdot}$ ($PhCH(OO^{\cdot})OH$), according to the Scheme 2 [20, 24].

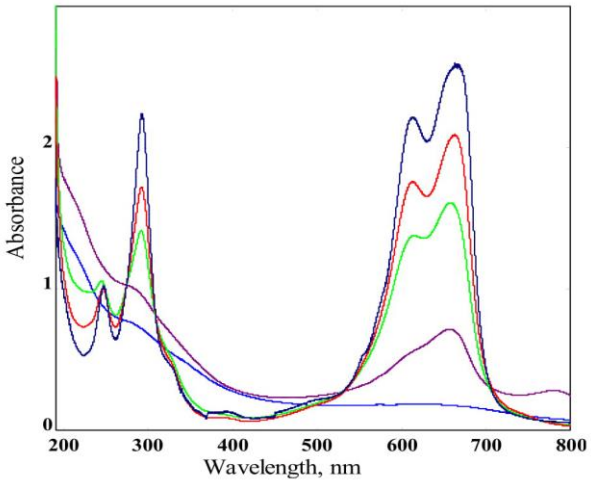


Figure 3. UV-visible spectra of MB solution with concentration of $3 \cdot 10^{-5}$ mol/l (dark blue line) and MB solutions after 10 min treatment with Fenton system at different molar ratio of MB to $FeSO_4$: red line – 1:1, green line – 1:3, violet line – 1:5, blue line – 1:10 (t = 20 ºC)

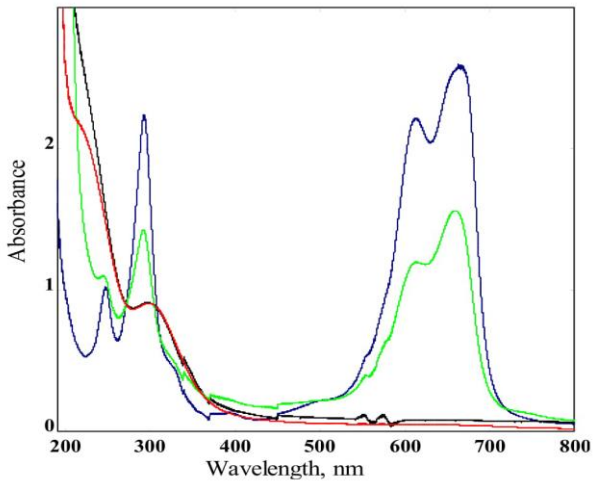


Figure 5. UV-visible spectra of MB solution with concentration of $3 \cdot 10^{-5}$ mol/l (dark blue line) and MB solutions after 20 min treatment with Fenton system ($FeSO_4$ (C = $3 \cdot 10^{-4}$ mol/l) + H_2O_2 (C = $4.4 \cdot 10^{-3}$ mol/l) (red line); black line - in the presence of guanidine hydrochloride (C=$3 \cdot 10^{-4}$ mol/l), green line – PHMG-Cl (C=$3 \cdot 10^{-4}$ mol/l) (t = 20 ºC)

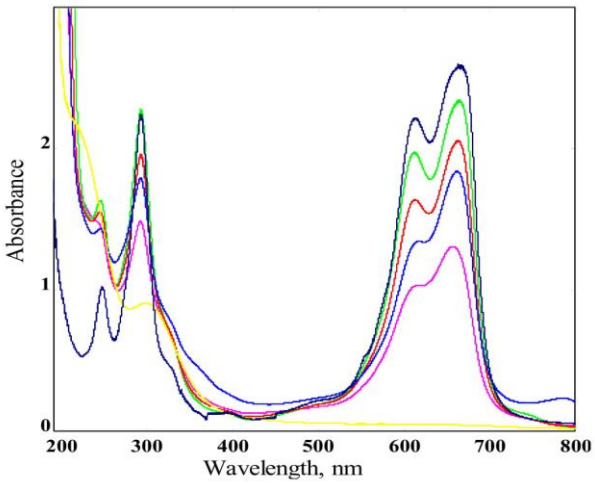


Figure 4. UV-visible spectra of MB solution (C=$3 \cdot 10^{-5}$ mol/l, dark blue line) and MB solutions after 20 min treatment with Fenton system ($FeSO_4$ (C = $1.5 \cdot 10^{-4}$ mol/l) + H_2O_2 (C = $2.2 \cdot 10^{-3}$ mol/l) in the presence of different concentrations of PHMG-Cl: $9 \cdot 10^{-4}$ mol/l (green line), $6 \cdot 10^{-4}$ mol/l (red line), $3 \cdot 10^{-4}$ mol/l (blue line), $1.5 \cdot 10^{-4}$ mol/l (pink line), $3 \cdot 10^{-5}$ mol/l (yellow line) (t = 20 ºC)

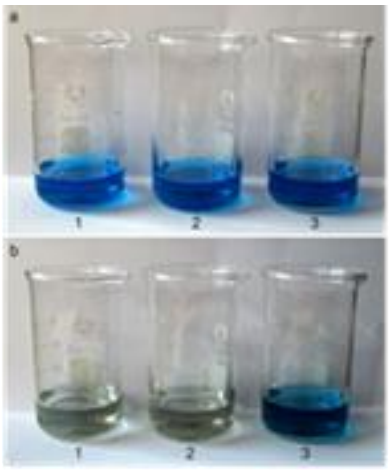


Figure 6. MB solutions (C=$3 \cdot 10^{-5}$ mol/l) before (a) and after 20 min treatment with Fenton system ($FeSO_4$ (C = $3 \cdot 10^{-4}$ mol/l) + H_2O_2 (C = $4.4 \cdot 10^{-3}$ mol/l) (b): 1 – control solution, 2 – solution containing guanidine hydrochloride (C=$3 \cdot 10^{-4}$ mol/l), 3 – solution containing PHMG-Cl (C=$3 \cdot 10^{-4}$ mol/l) (t = 20 ºC)

Kinetic curves of oxygen absorption by BA in the presence of PHMG-Cl are shown in Fig. 7. The pure model system BA-AIBN absorbed oxygen with the rate W = $4.21 \cdot 10^{-6}$ mol/(l·s). The introduction of PHMG-Cl into the oxidized system in the concentrations ranged from $2.3 \cdot 10^{-2}$ g/100 ml to $2.92 \cdot 10^{-1}$ g/100 ml decreased the oxidation rate to $4.02 \cdot 10^{-6}$ mol/l·s (by 4.51 %) and to $0.49 \cdot 10^{-6}$ mol/l·s (by 88 %), respectively. With further increase of initial concentration of PHMG-Cl the oxidation rate of BA tended to its limit value W_∞ (Fig. 8). The chain length of stabilized oxidation (W_∞ / W_i) is found to be 16 that means the process proceeded in a chain mode. Overall, the obtained data indicate the antioxidant activity of PHMG-Cl since it effectively inhibits radical chain oxidation of BA.

In most cases the inhibition of free radicals proceeds via hydrogen cleavage from antioxidant molecule AH [21]:

$$RO_2^{\cdot} + AH \longrightarrow ROOH + A^{\cdot}$$

Based on the literature data, one can assume that PHMG-Cl may interact with free radicals similar to tertiary amines, via α-C-H bond rupture to α-amino radical [25].

The α-amino intermediate is then intercepted by an appropriate reagent to furnish a new α-C-R bond. Following the general scheme of inhibition of free radicals by PHMG-Cl could be suggested (Scheme 3).

Thus, the results of this study clearly demonstrated the antioxidant activity of PHMG-Cl in the concentration range from $3\cdot10^{-4}$ to $3\cdot10^{-2}$ mol/l at which it also shows antimicrobial activity [3-6, 8]. Moreover, PHMG-Cl has been reported to stimulate plant growth, as well as significantly improve copper stress resistance of plants in the concentration range from $5\cdot10^{-4}$ - $5\cdot10^{-3}$ mol/l [17]. It is well known that the excess of copper salts in plant cells impairs the redox homeostasis due to the generation of reactive oxygen species - superoxide-anions, hydroxyl radicals, hydrogen peroxide, causing lipid peroxidation in cells and thus enhancing non-specific permeability of membranes [26]. Therefore, the authors supposed that the antioxidant activity of PHMG-Cl may be the main factor defining the mitigation of the toxic effect of copper ions on wheat seedlings [17].

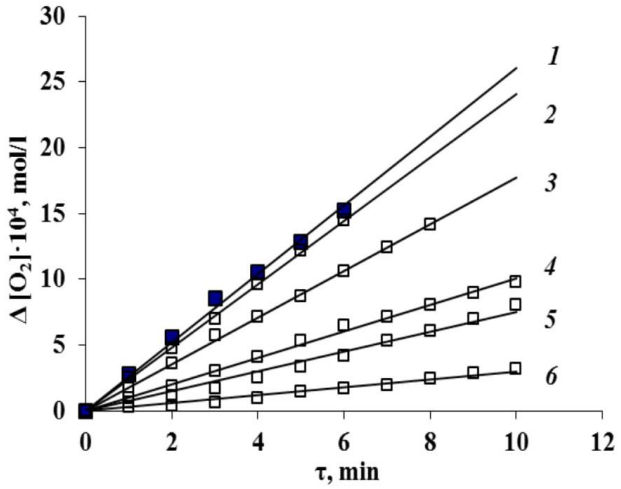


Figure 7. Oxygen absorption kinetic in the model system of initiated oxidation of benzyl alcohol without inhibitor (1) and in the presence of different concentrations of PHMG-Cl: 0.023 g/100 ml (2), 0.073 g/100 ml (3), 0.146 g/100 ml (4), 0.234 g/100 ml (5), 0.292 g/100 ml (6) ($W_i = 2.98\cdot10^{-8}$ mol/(l·s), 50 °C)

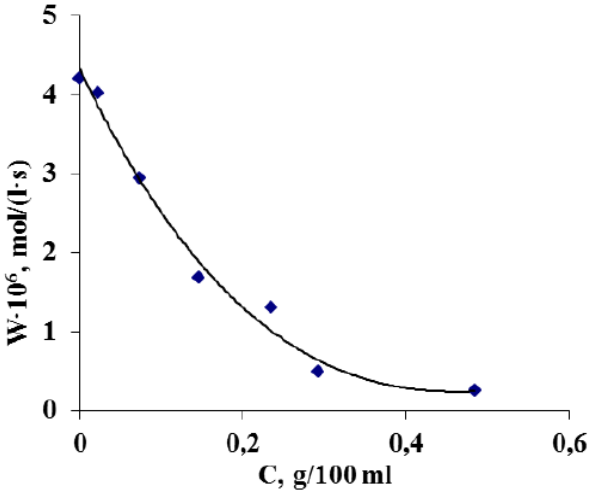
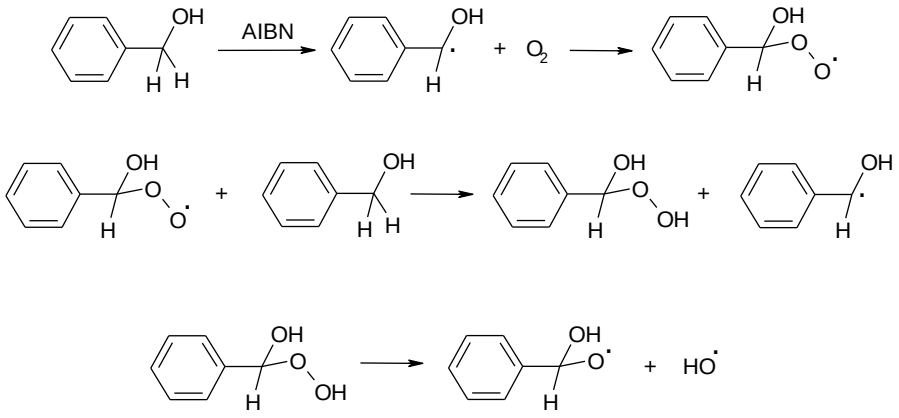
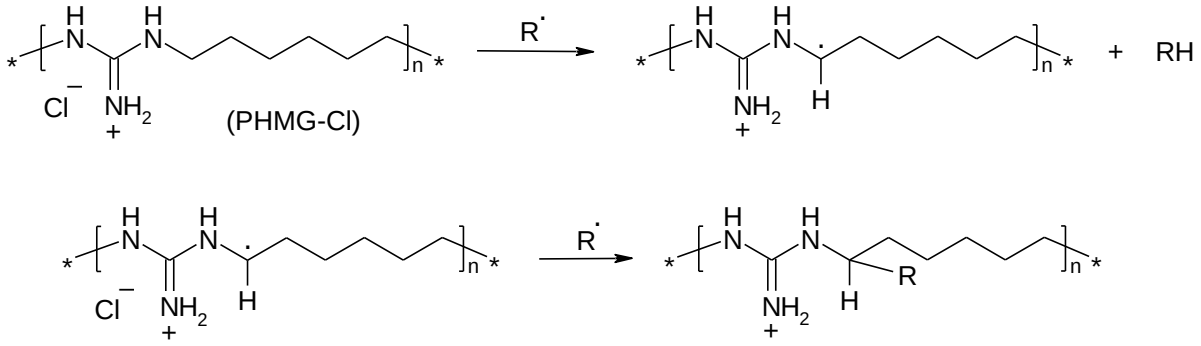


Figure 8. The rate of inhibited oxidation of benzyl alcohol versus initial concentration of PHMG-Cl ($W_i = 2.98\cdot10^{-8}$ mol/(l·s), 50 °C)



Scheme 2. Initiated oxidation of benzyl alcohol



Scheme 3. Possible mechanism of free radical inhibition by PHMG-Cl

Conclusions

The antioxidant activity of polymeric biocide PHMG-Cl has been established by using different methods. In the methylene blue (MB) dye test, the oxidation of MB by hydroxyl radicals generating in Fenton system was found to decrease in the presence of PHMG-Cl in molar ratio to MB of 5:1 and 10:1 (by 26 % and 38 %, respectively). At the same time, complete dye oxidation was observed when guanidine hydrochloride was used instead of PHMG-Cl. The last fact indicates that alkyl substituents near guanidinium cation are necessary for the hydroxyl radical scavenging activity.

The antioxidant activity of PHMG-Cl has also been studied in the model system of radical chain oxidation of benzyl alcohol (BA). In this system, alkyl and peroxyl radicals are formed. Under experimental conditions, antioxidant activity was determined by a decrease in the initial rate of oxygen absorption during the initiated oxidation of BA. It has been found that the introduction of PHMG-Cl into the oxidized system in the concentrations ranged from $2.3 \cdot 10^{-2}$ g/100 ml to $2.92 \cdot 10^{-1}$ g/100 ml decreased the oxidation rate of BA by 4.5–88 %. The process proceeds in a chain mode. This result demonstrates that PHMG-Cl effectively inhibits radical chain oxidation of BA. Based on literature data for free radical scavenging activity of alkylguanidine derivatives, it can be assumed that the interaction of PHMC-Cl with radical species (R) proceeds through α-C-H bond hemolysis followed by the formation of a new α-C-R bond.

Conflicts of Interest

The authors declare no conflict of interest.

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Антиоксидантна активність полімерного біоциду полігексаметиленгуанідин гідрохлориду

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Катіонний полімер полігексаметиленгуанідин гідрохлорид (ПГМГ-Cl) є перспективним біоцидом, який має широкий спектр антимікробної активності, помірну токсичність, а також невисоку вартість. Він застосовується як дезінфектант в системах охолодження, плавальних басейнах і медичних закладах, засобах особистої гігієни і т. д. Нещодавно встановлено, що ПГМГ-Cl має виражені протизапальні та ранозагоювальні властивості і є ефективним для обробки хронічних ран та термічних опіків. Це може свідчити про антиоксидантну активність полімерного біоциду.

В цій роботі синтезовано ПГМГ-Cl поліконденсацією гідрохлориду гуанідину та гексаметилендіаміну у розплаві. Будову катіонного полімеру підтверджено методами ^{1}H ЯМР спектроскопії та ІЧ-спектроскопії. Визначено середнов'язкісну молекулярну масу ПГМГ-Cl, яка становить 10700.

Вивчено антиоксидантну активність ПГМГ-Cl різними методами. Встановлено, що окиснення барвника метиленового синього (МС) гідроксильними радикалами, які генеруються в системі Фентона, суттєво сповільнюється за присутності ПГМГ-Cl у мольному співвідношенні до МС 5:1 і 10:1 (на 26 % і 38 % відповідно). В той же час, при використанні гідрохлориду гуанідину замість полімерного біоциду відбувалось повне окиснення барвника. Досліджено антиоксидантну активність ПГМГ-Cl в модельній системі радикально-ланцюгового окиснення бензилового спирту (БС) під дією генерованих алкільних і пероксильних радикалів. Активність полімерного біоциду проти вільних радикалів визначали за зменшенням початкової швидкості поглинання кисню при ініційованому окисненні БС. Встановлено, що введення ПГМГ-Cl в окиснювальну систему в області концентрацій $1.3\cdot10^{-3}$ - $1.6\cdot10^{-2}$ моль/л зменшує швидкість окиснення субстрату на 4.5-88 %. Отримані результати свідчать про здатність катіонного полімеру ефективно інгібувати радикально-ланцюгове окиснення БС. Однак для встановлення механізму дезактивації вільних радикалів полімерним біоцидом необхідне проведення наступних досліджень.

Ключові слова: полігексаметиленгуанідин, полімерний біоцид, антиоксидантна активність, метиленовий синій, радикально-ланцюгове окиснення, поглинання кисню