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Synthesis and biocidal activity of novel *N*-halamine hydantoin-containing polystyrenes

Abstract: Three homopolymers containing hydantoin substituents were obtained by chemical modification of reactive *p*-chloromethylated polystyrene. The prepared polymers were chlorinated to yield *N*-halamine materials with biocidal properties. The chemical structure of polymers was characterized by Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. All of the hydantoin polymers are insoluble in water and common organic solvents. Microbiological investigations prove the high biocidal activity of the obtained chlorinated polystyrene derivatives containing spirohydantoin moieties. The obtained polymers will be useful in designing and constructing medical and pharmaceutical equipment. The ability to crosslink allows to expect easy grafting of these biocidal macrochains, for example, on textiles.

Keywords: biocidal activity; biocidal polymers; *N*-halamine; polymer characterization; polymer synthesis.

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1 Introduction

Vinyl monomers are a large family of compounds used currently in polymerization on a big, industrial scale. Chemical modification of such monomers by introduction of new substituents allows to obtain materials with desired properties for special applications: polymers for surgery, agriculture and packaging industry, thermoresistant and non-flammable protective coatings (in architecture, means of transport) as well as plastics with extra high mechanical properties, excellent swelling ability (hydrogels) or lack of wettability (e.g., anti-adhesive, self-cleaning layers) (1–6). The latest publications have demonstrated that control of polymerization mechanism and kinetics also enables the manipulation of polymer structure and properties.

Recently, a great deal of interest is being devoted to biocidal polymers, which can be used for the production of textiles able to kill pathogenic microorganisms or at least that are characterized with high resistance to microbial attack. Such promising properties have polymers based on *N*-halamines – compounds containing oxidative halogen attached to nitrogen atom (7–12). The bacteriocidal action of these compounds involves the transfer of halogen, directly to microorganism cells. It destroys the metabolic and enzymatic processes in cells and, in consequence, leads to their necrosis. An important advantage of *N*-halamine polymers is the possibility of regeneration of their biocidal properties after long-term usage and washing. The biological activity can be recovered in a simple halogenation process.

N-Halamine polymers can be used in the textile industry, in the production of water or air filters, hygienic articles and protective coatings in hospital equipment, bathroom accessories or covers of ships. Numerous examples of *N*-halamine polymers have been described recently (7–21). Various amines, amides and imides have been treated by hypochlorous acid or its salt (hypochlorite) and grafted onto natural (mainly cellulose/cotton) or synthetic (polyester, polyamide) fibers (13, 14, 22–27). Such modification of polymers led to satisfactory disinfective action for a broad range of microorganisms.

N-Halamines can be obtained from hydantoins, which represent a group of compounds characterized by diverse biological activities including antibacterial, antiviral, fungicidal, herbicidal, etc. A special kind of hydantoins

is spirohydantoins, which bond chemically to alicyclic ring condensed with aromatic ring. Spirohydantoins were found to have high biological and anti-inflammatory activities (28).

The aim of this work was to prepare new polystyrene-based polymers containing three different hydantoin moieties (including two spirohydantoin groups) using *N*-alkylation reaction. The obtained spirohydantoin-containing polymers were then chlorinated, thanks to the presence of reactive N-H bond, which was easily transformed into N-Cl. The chemical structure of polymers after hydantoin substitution and chlorination was characterized by nuclear magnetic resonance (NMR) and/or Fourier transform infrared (FTIR) spectroscopy. The biocidal activity of novel polymers against *Staphylococcus aureus* and *Escherichia coli* has been investigated. For understanding the mechanism of biocidal action, the content of chlorine evolved from polymer has been estimated according to a standard procedure.

2 Results and discussion

2.1 Preparation of polymers

Chloromethylated polystyrene, prepared via bulk polymerization of 4-vinylbenzyl chloride, was used for the synthesis of materials containing hydantoin moieties. The way of reaction is presented in Figure 1.

The first step of this synthetic approach (polymerization) was completed after 2 h. The white product was rinsed by ethanol for complete removal of the monomer traces and other impurities. Finally, the product was dried at 50°C in a vacuum for 24 h. The yield of the obtained *p*-chloromethylated polystyrene (MPS-Cl) was almost 80%.

The average molecular weight and polydispersity of MPS-Cl were 26,700 Da and 2.5, respectively.

Although the functionalization of *p*-chloromethylated polystyrene has been described as a good method of preparation of methylated polystyrenic hydantoin beads, the yield of the reaction and conditions need to be improved (29). In the described method, the potassium salt of hydantoin (prepared by the reaction of hydantoin with potassium hydroxide in boiling ethanol) reacted with *p*-chloromethylated polystyrene in dimethylformamide (DMF) solution at 100°C for 12–48 h. Nevertheless, some problems in the purification of the final product have been observed. For the purpose of improving this preparation method, we decided to develop the *N*-alkylation reaction.

The reactive polymer MPS-Cl was added to a stirred DMF suspension of hydantoin and potassium carbonate. After overnight stirring at room temperature, the polymer was precipitated by water addition. The product was isolated by filtration and washed with ethanol and warm water to remove the solvent. Finally, the polymer was dried under vacuum at 50°C overnight.

This reaction appeared to be a useful technique for the functionalization of *p*-chloromethylated polystyrene and for the synthesis of hydantoin-containing polymers (Figure 1), which were the precursors of biocidal polymers. To transform these polymers into *N*-halamine, the appropriate hydantoin-containing polymers were stirred for 30 min at room temperature with trichloroisocyanuric acid (TClCA) in acetone, during which N-H bond was transformed into N-Cl.

This synthetic process has been used several times for the preparation of all three polymers with the same results.

All polymers were characterized as totally insoluble in water and common organic solvents. It can be supposed that this is the result of a strong intramolecular and intermolecular hydrogen bond formation between hydantoin rings or crosslinking by covalent bonds.

Because the polymers were insoluble either in chloroform, dimethylsulfoxide or tetrahydrofuran (even on heating), their ^{1}H NMR spectra and exact molecular

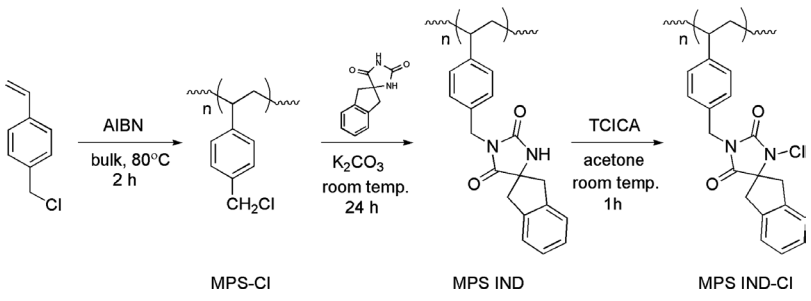

Figure 1 Scheme of the synthesis of *N*-halamine hydantoin-containing polystyrenes.

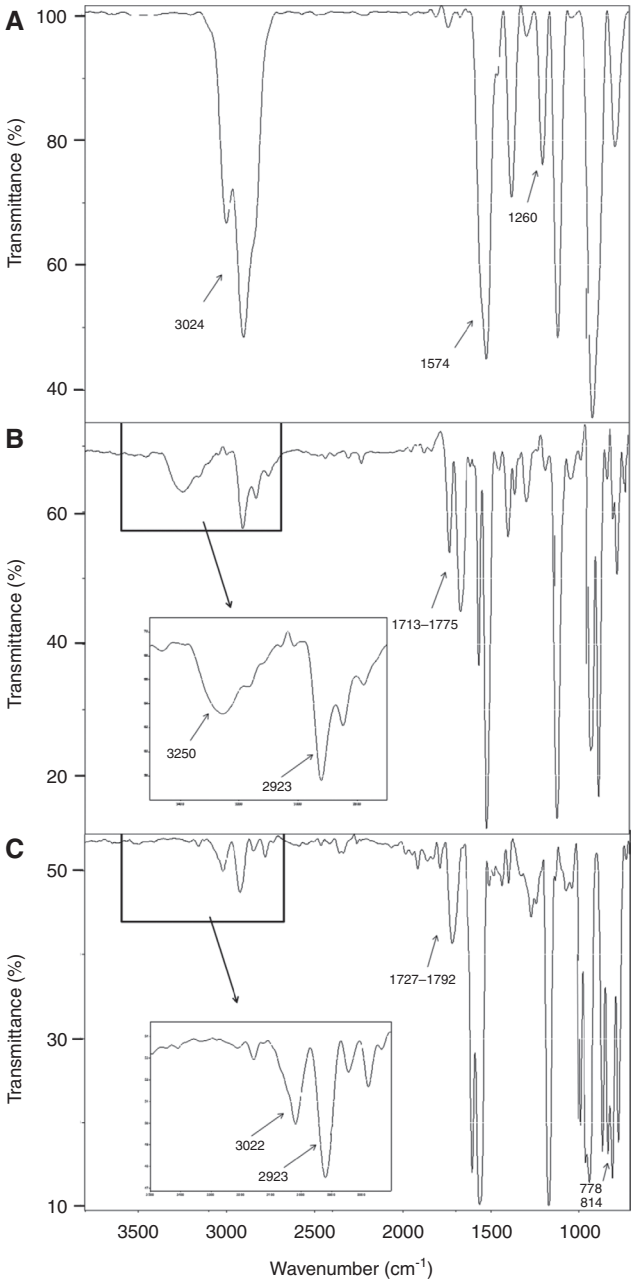

Figure 2 The FTIR spectra of (A) MPS-Cl, (B) MPS TET, and (C) MPS TET-Cl.

weights therefore cannot be measured. For confirmation of the expected structures of the obtained polymers, ^{13}C NMR in solid-state and FTIR [in hexachloro-1,3-butadiene suspension (HCBD)] spectra were done.

2.2 FTIR and NMR characteristics of the polymers

FTIR spectra of the newly obtained polymers were very similar. Therefore, only the spectra of tetralone polymers (MPS-TET and MPS TET-Cl) are shown as examples and compared to the spectrum of MPS-Cl (Figure 2).

In chloromethylated polystyrene (MPS-Cl) spectrum, the characteristic bands at 2923 and 1574 cm^{-1}, assigned to the C-H stretching and C-C skeleton vibrations of the aromatic ring of polystyrene, have been observed (30). The presence of the CH_2-Cl group of chloromethylated PS was confirmed by the strong band at 1260 cm^{-1} attributed to the stretching vibrations of this moiety (Figure 2A). This band disappeared in the spectra of polymers containing hydantoins. Simultaneously, two new bands appeared at 1780 and 1711 cm^{-1} for MPS DMH; 1778 and 1689 cm^{-1} for MPS IND; and 1775 and 1713 cm^{-1} for MPS TET polymer (Figure 2B). They can be assigned to the C=O stretching vibrations of the hydantoin groups. In accordance with the literature data, hydantoin spectra exhibit two absorption bands connected with the stretching vibrations of the carbonyl groups due to the keto-enol tautomerization of these compounds (amide and imide structures) (31).

The characteristic wide bands at around 3300 cm^{-1} were attributed to the N-H stretching vibrations of the hydantoin rings, indicating a successful coupling reaction (Figure 2B).

The spectrum in Figure 2C was obtained after the chlorination of the polystyrene containing the attached hydantoin groups. The band near 3300 cm^{-1} had disappeared, and two new bands at 814 and 778 cm^{-1}, assigned to the N-Cl groups, were detected. It indicates the successful chlorination of the hydantoin ring. Furthermore, the transformation of N-H bonds to N-Cl groups is associated with the breakage of C=O••••H-N hydrogen bonding. This caused the shift of the C=O bands from 1775 and 1713 cm^{-1} in MPS TET to 1792 and 1727cm^{-1} in MPS TET-Cl, respectively. The low intensive, unresolved doublet at carbonyl bands can be assigned to the coupled C=O/C-N vibrations. This band is shifted to higher frequency after chlorination by TClCA (32).

All these observations proved the introduction (substitution) of hydantoin rings instead of chlorine atoms in the macrochain of *p*-chloromethylated polystyrene via *N*-alkylation reaction.

For more precise determination of the structures of the synthesized compounds, NMR spectroscopy was used.

The ^1H NMR spectra of hydantoins confirmed the chemical structures of these compounds. All spectra of hydantoins [5,5-dimethylimidazolidine-2,4-dione (DMH), 1′,3′-dihydrospiro[imidazolidine-4,2′-indene]-2,5-dione (IND), and 7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione (TET)] show two singlets at around 8.5 and 10.5 ppm attributed to protons from the N-H groups.

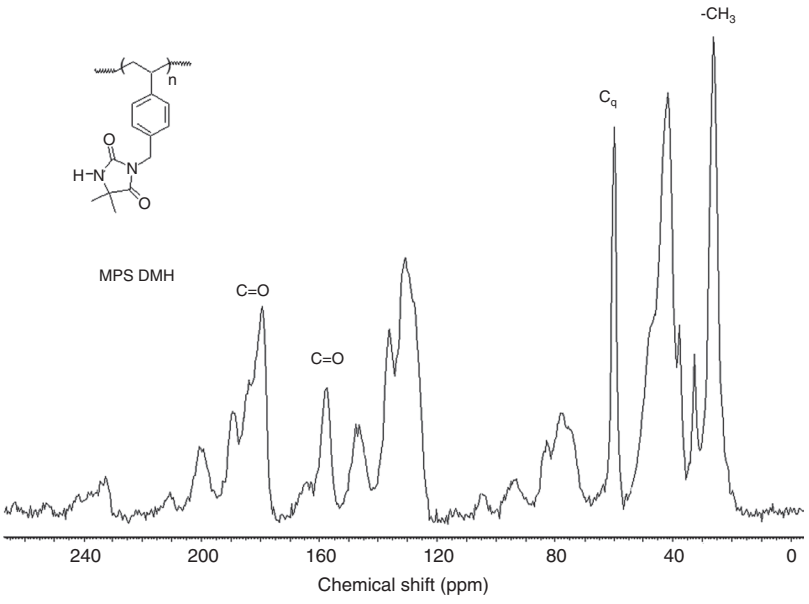

Figure 3 Solid-state ^{13}C NMR spectrum of MPS DMH.

In the ^{13}C NMR spectra of all hydantoins (DMH, IND, and TET), the characteristic peaks at 156 and 178 ppm, attributed to the carbonyl groups of the hydantoin ring, were observed.

The ^{1}H NMR spectra of soluble *p*-chloromethylated polystyrene (MPS-Cl) were well consistent with the expected structure. The weak peaks at around 4.5 and 5.5 ppm were attributed to vinyl groups at the ends of the polymer but not to unreacted monomer (which was removed completely by washing with ethanol).

In all hydantoin-containing polystyrenes (MPS DMH, MPS IND, and MPS TET), such peaks at around 156 and 177 ppm were detected. The presence of these peaks confirms that substitution reaction in chloromethylated PS takes place.

The other peaks of the spectra were also very consistent with the proposed structures of the hydantoin-containing polymers (an example is shown in Figure 3).

2.3 Biocidal activity

The results of the biocidal activity of the tested substances are presented in Tables 1–3.

As can be seen, all tested *N*-halamine polymers show the biocidal properties dependent on the chemical structure of hydantoin. The tested polymers can be ordered according to the growing bioactivity as follows:

MPS IND-Cl<MPS DMH-Cl<MPS TET-Cl

MPS TET-Cl, even in the lowest applied concentration, eliminates the all viable bacteria *S. aureus* within 15 min,

Table 1 Results of the determination of the antibacterial effect of **MPS DMH-Cl**.

Contact time (min)	Concentration (mg/cm³)				
	0.5	**1**	**5**	**10**	**25**
Staphylococcus aureus (n=1.5×10⁸)					
5					
N_a	115	5	0	0	
R	1.3×10⁵	3.0×10⁶	Total	Total	Total
15					
N_a	20	1	0	0	0
R	7.5×10⁵	1.5×10⁷	Total	Total	Total
60					
N_a	5.5	0	0	0	0
R	2.7×10⁶	Total	Total	Total	Total
Escherichia coli (n=1.7×10⁸)					
5					
N_a	>1000	120	0	0	0
R	<1.7×10⁴	1.4×10⁵	Total	Total	Total
15					
N_a	23	14	0	0	0
R	7.4×10⁵	1.2×10⁶	Total	Total	Total
60					
N_a	5.5	8.5	0	0	0
R	3.1×10⁶	2.0×10⁶	Total	Total	Total

n, Number of living bacteria in the tested bacterial suspension (CFU/cm³); N_a, average number of living bacteria in the bacterial suspension exposed to MPS DMH-Cl (CFU/cm³); R, reduction in the number of viable bacteria.

Table 2 Results of the determination of the antibacterial effect of MPS IND-Cl.

Contact time (min)	Concentration (mg/cm³)				
	0.5	1	5	10	25
Staphylococcus aureus (n=1.5×10⁸)					
5					
N_a	1500	500	107	0	0
R	1.0×10⁴	3.0×10⁴	1.4×10⁵	Total	Total
15					
N_a	500	395	15	0	0
R	3.0×10⁴	3.8×10⁴	1.0×10⁶	Total	Total
60					
N_a	81	91	7,5	0	0
R	1.8×10⁵	1.6×10⁵	2.0×10⁶	Total	Total
Escherichia coli (n=1.7×10⁸)					
5					
N_a	1.5×10⁸	155	150	0	0
R	0.1	1.1×10⁵	1.1×10⁵	Total	Total
15					
N_a	6.4×10³	120	135	0	0
R	2.3×10³	1.4×10⁵	1.2×10⁵	Total	Total
60					
N_a	155	72	125	0	0
R	1.1×10⁵	2.6×10⁵	1.4×10⁵	Total	Total

Explanations of n, N_a, and R as in Table 1.

Table 3 Results of the determination of the antibacterial effect of MPS TET-Cl.

Contact time (min)	Concentration (mg/cm³)				
	0.5	1	5	10	25
Staphylococcus aureus (n=1.5×10⁸)					
5					
N_a	4.5	4	0	0	0
R	3.3×10⁶	3.4×10⁶	Total	Total	Total
15					
N_a	0	0	0	0	0
R	Total	Total	Total	Total	Total
60					
N_a	0	0	0	0	0
R	Total	Total	Total	Total	Total
Escherichia coli (n=1.7×10⁸)					
5					
N_a	5	1.5	0	0	0
R	3.4×10⁶	1.1×10⁷	Total	Total	Total
15					
N_a	1	0	0	0	0
R	1.7×10⁷	Total	Total	Total	Total
60					
N_a	0	0	0	0	0
R	Total	Total	Total	Total	Total

Explanations of n, N_a, and R as in Table 1.

whereas *E. coli* within 60 min (Table 3). MPS IND-Cl in the 0.5 mg/cm³ concentration and 5 min of contact time causes a very small (almost invisible) reduction of viable bacteria (Table 2). The concentration of 5 mg/cm³ of MPS TET-Cl and MPS DMH-Cl was sufficient to inactivate all viable bacteria even during the shortest time of the contact considered (5 min). Thus, longer contact time is not needed for total killing of the tested microorganisms (Tables 1 and 3).

In the case of MPS IND-Cl, the lowest concentration causing a total elimination of living bacteria in 5 min is 10 mg/cm³.

As expected, the reduction in the number of living bacteria (*R* value) increases with the increase in the concentration and the contact time for all tested substances.

All samples provided a potential biocidal function against both species, but a different sensitivity of the tested bacterial strains during contact with the polymers studied was observed. Definitely, the most sensitive to biocidal action of all tested substances was a Gram-positive strain of *S. aureus*. It can be related to the dissimilarities in structure of these bacteria. The main difference is that the cell wall of Gram-negative bacteria is overlaid with the outer layer. This is a complementary layer, which limits or prevents the penetration of biocidal agent into the cell (33). Thus, inactivation of *E. coli* demands a longer contact time than that for *S. aureus*.

2.4 Concentration of chlorine in aqueous extracts

It has been reported that the biocidal properties of *N*-halamines are a result of the direct transport of Cl^+ into bacteria cells (33). This reaction can wreck or inhibit metabolic and enzymatic cell processes.

However, to understand better the mechanism of biocidal action of the studied polymers, it was important to check the role of chlorine. We took into account the possibility of chlorine evolution from polymers in an aqueous environment.

For this purpose, the polymers were immersed in distilled water, and after 30 min, the content of free chlorine was immediately measured. The observed concentration of Cl_2 was very small (0.13–0.22 mg/l; Table 4). According to literature data, such concentration is insufficient to cause disinfection (23). This experiment indicates that the obtained *N*-halamine polymers are stable in a water environment.

Interestingly, the amount of chlorine found in aqueous extracts taken after the biocidal tests was much

Table 4 Concentration of free chlorine (mg/l) in aqueous extracts from chlorinated hydantoin-containing polystyrenes.

Polymer	Extract from polymer	Extract from polymer after biocidal test with *S. aureus*	Extract from polymer after biocidal test with *E. coli*
MPS DMH-Cl	0.22	136.0	56.0
MPS IND-Cl	0.20	70.0	20.0
MPS TET-Cl	0.13	122.0	68.0

higher (few tens in the case of *E. coli* and even higher than 100 in the case of *S. aureus*) despite the precise washing of samples before the biocidal test (Table 4).

Furthermore, the amount of released chlorine in extracts after a biocidal test with various density of bacteria suspension was also determined (Table 5). In most cases, the bigger the density of the suspension of bacteria, the higher the chlorine concentration. Only MPS IND-Cl did not show such trend.

The lower biocidal activity of MPS IND-Cl is probably related to the stabilization of the N-Cl bond due to the delocalization of the electron. The stability in the compound with the alicyclic ring condensed with benzene ring is similar as in the well-known homoaromatic compounds. In addition, this delocalized bond interacts with the free electron pair of the nitrogen atom. Thus, the N-Cl bond is simultaneously stabilized by the conjugation (34).

It seems probable that the presence of bacteria in medium stimulates the secretion of chlorine from the polymer. This means that evolved chlorine is also responsible for bacteria killing in the studied conditions. This finding provides new information and leads to additional explanation of the mechanism of bacteria apoptosis in the presence of *N*-halamine polymers.

Comparing the amount of released chlorine with the biocidal efficacy of polymers, we noticed that the halogen present in the solution indeed affected the biocidal action of the investigated polymers. The lowest concentration of chlorine was found in the MPS IND-Cl sample, which was characterized by the lowest antibacterial action. These results confirm the previously suggested hypothesis that conjugation with the pseudo-homoaromatic structure of indanone influences the stabilization of the N-Cl bond.

Table 5 Concentration of free chlorine (mg/l) in an aqueous extract from chlorinated hydantoin-containing polystyrenes after a biocidal test in solution with different density values of the suspension of bacteria.

Polymer	Extract from polymer after biocidal test with *S. aureus*		Extract from polymer after biocidal test with *E. coli*	
	0.4	0.8	0.4	0.8
MPS DMH-Cl	20.5	24.5	34.0	14.0
MPS IND-Cl	3.0	2.0	1.0	1.0
MPS TET-Cl	96.0	525.0	19.0	44.0

3 Conclusions

Hydantoin-containing polystyrenes have been successfully synthesized by modification of the reactive polymer (MPS-Cl) in the simple and efficient substitution reaction. These polymers can be halogenated by the previously reported method for obtaining new *N*-halamine biocidal polymers.

All of the received compounds were characterized by FTIR and NMR spectroscopy, and in all cases the spectra were found to be consistent with the expected structures.

Chlorinated hydantoin-polystyrenes (*N*-halamines) exhibit high biocidal activity against *S. aureus* and *E. coli* even after a short contact time. MPS TET-Cl is characterized by higher biocidal activity than MPS DMH-Cl (which is known in the literature and used in the industry).

The evolution of chlorine is efficiently accelerated in the presence of living microorganisms; thus, it can be concluded that chlorine is an additional disinfection agent in studied systems.

The good biocidal properties of the described materials allow to predict their application in lower concentrations, for example, as active components in blends and composites or as antibacterial coatings. All of the obtained polymers are insoluble in water and common organic solvents. This is an advantage from a practical point of view, because the described materials can be used as active modifiers for polymers applied as a filter and also in medicine, pharmacy, or cosmetics. Moreover, the ability to crosslink allows to expect easy grafting of these biocidal macrochains on textiles. The lower the concentration of active polymeric modifier, the less expensive the biocidal plastics or fibrae.

4 Experimental part

4.1 Materials

Ketones (acetone, 2-indanone, 2-tetralone), 4-vinylbenyl chloride, potassium cyanide, ammonium carbonate, potassium carbonate anhydrous, azobisisobutyronitrile

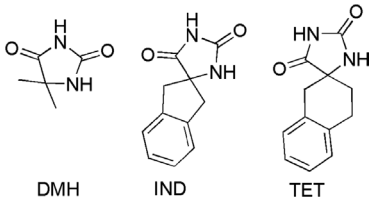

Figure 4 Chemical structures of the applied hydantoins.

(AiBN), TClCA, 10 polystyrene standards, and all solvents used in this research were purchased from Sigma Aldrich (Saint Louis, MO, USA). All reagents were pure per analysis and were used without further purification.

4.2 Synthesis of hydantoins

DMH), IND, and TET (Figure 4) were obtained from appropriate ketone by the Bucherer-Bergs reaction (35).

4.3 Preparation of the precursor polymer: *p*-chloromethylated polystyrene (MPS-Cl; Figure 2)

The AiBN (30 mg) was added carefully to 4-vinylbenzyl chloride (1.53 g, 10 mmol) at 80°C, under nitrogen atmosphere in three portions over 20 min. After 2 h, the mixture became solid and the polymer was cooled to ambient temperature and washed several times washed with ethanol (20 ml), water (20 ml) and ethanol again. The product was dried overnight at 50°C. MPS-Cl was obtained as a white solid (1.25 g) with 80% yield.

IR (HCBD): ν 3024, 2923, 1574, 1260 cm^{-1};

^{1}H NMR, (700 MHz), $CDCl_3$, δ (ppm), for repeat MPS-Cl moiety: 1.35–1.52 (m,CH_2), 1.69 (m, CH), 4.50 (m, CH_2-Cl), 6.42 (m, CH_{Ar}), 7.10 (m, CH_{Ar}); for vinyl terminations: 4.56 (s, =CH_2), 5.25–5.75 (m, =CH_2), 7.35 (d, J=8.4 Hz, CH_{Ar}), 7.38 (d, J=8.4 Hz, CH_{Ar}).

^{13}C NMR, (400 MHz), $CDCl_3$, δ (ppm), for repeat MPS-Cl moiety: 40.3 (CH), 46.1 (CH_2), 46.3 (CH_2), 128.5 (CH_{Ar}), 128.8 (CH_{Ar}), 134.9 (C_q), 145.5 (C_q); for vinyl terminations: 114.6 (=CH_2), 126.5 (CH_{Ar}), 127.8 (CH_{Ar}), 136.2 (C_q), 136.9 (C_q), 137.7 (C_q), 145.0 (C_q).

4.4 Preparation of hydantoin-containing polymers (Figure 1): *N*-alkylation reaction of chloromethylated polystyrene

Chloromethylated polystyrene (1.25 g) and potassium carbonate (10 mmol) were added to a suspension of hydantoin (10 mmol) in DMF (50 ml). The mixture was stirred overnight and then water (20 ml) was added. A white precipitate was formed and filtered off, several times rinsed with water and ethanol, and dried overnight at 50°C.

4.5 Poly(3-(4′-vinylbenzyl)-5,5-dimethylimidazolidine-2,4-dione) (MPS DMH)

Starting from DMH, this product was isolated as an insoluble white solid.

IR (HCBD): ν 3291, 2928, 1780, 1711 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 26 (CH_3), 41 (CH_2), 60 (CH_2), 77 (C_q), 114 (weak) (=CH_2), 128–130 (CH_{Ar}), 135–139 (C_q), 147 (CAr_q), 157 (C=O), 179 (C=O).

4.6 Poly(3-(4′-vinylbenzyl)-1′,3′-dihydrospiro[imidazolidine-4,2′-indene]-2,5-dione) (MPS IND)

Starting from IND, this product was isolated as a white insoluble solid.

IR (HCBD): ν 3274, 3023, 1778, 1689 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 37–42.8 (CH_2), 71.6 (CH_2), 78.0 (C_q), 114.6 (weak) (=CH_2), 126.5–127.0 (CH_{Ar}), 135.0–140.0 (C_q), 146 (C_{Arq}), 157.1 (C=O), 178.4 (C=O).

4.7 Poly(3-(4′-vinylbenzyl)-7,8-Benzo-1,3-diazaspiro[4.5]decane-2,4-dione) (MPS TET)

Starting from TET, this product was isolated as a white insoluble solid.

IR (HCBD): ν 3250, 2923, 1775, 1713 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 26–37.4 (CH_2), 41.3 (CH_2), 61.7 (CH_2), 76.5 (C_q), 114.6 (weak) (=CH_2), 127–129.0 (CH_{Ar}), 135.0–140.0 (C_q), 145.6 (C_{Arq}), 157.3 (C=O), 179.9 (C=O).

The chemical structures of the obtained hydantoin-containing polymers are shown in Figure 5 (where X=H).

4.8 Chlorination of hydantoin-containing polymers

Trichloroisocyanuric acid (15 mmol) was added to the polymer solution (5 mmol) in acetone (10 ml), and the mixture was stirred at room temperature. After 30 min, the

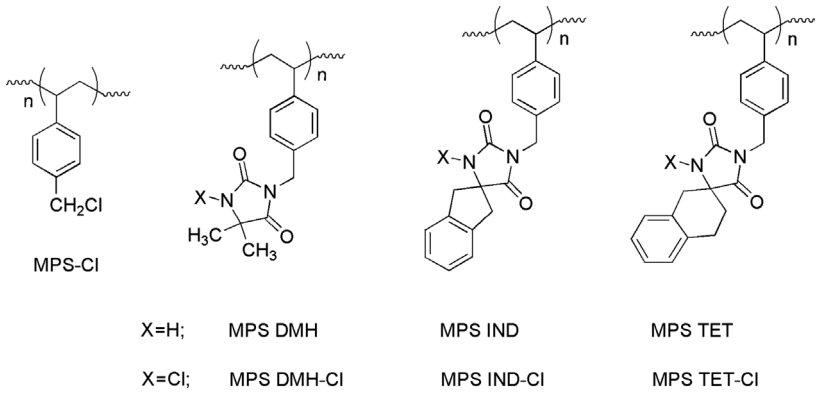

Figure 5 Chemical structures of the obtained polymers.

solvent was removed by evaporation. The obtained white powder was rinsed by hexane several times. The solid was separated by filtration and dried overnight at 40°C.

4.9 *N*-Chlorinated poly(3-(4′-vinylbenzyl)-5,5-dimethylimidazolidine-2,4-dione) (MPS DMH-Cl)

Starting from DMH, this product was isolated as an insoluble white solid.

IR (HCBD): ν 3092, 2928, 1791, 1729, 816, 774 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 26 (CH_3), 41 (CH_2), 60 (CH_2), 77 (C_q), 114 (weak) (=CH_2), 128–130 (CH_{Ar}), 135–139 (C_q), 147 (C_{Arq}), 157 (C=O), 179 (C=O).

4.10 *N*-Chlorinated poly(3-(4′-vinylbenzyl)-1′,3′-dihydrospiro[imidazolidine-4,2′-indene]-2,5-dione) (MPS IND-Cl)

Starting from IND, this product was isolated as a white insoluble solid.

IR (HCBD): ν 3034, 2922, 1782, 1711, 820, 774 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 37–42.8 (CH_2), 71.6 (CH_2), 78.0 (C_q), 114.6 (weak) (=CH_2), 126.5–127.0 (CH_{Ar}), 135.0–140.0 (C_q), 146 (C_{Arq}), 157.1 (C=O), 178.4 (C=O).

4.11 *N*-Chlorinated poly(3-(4′-vinylbenzyl)-7,8-benzo-1,3-diazaspiro[4.5]decane-2,4-dione) (MPS TET-Cl)

Starting from TET, this product was isolated as a white insoluble solid.

IR (HCBD): ν 3022, 2923, 1792, 1727, 814, 778 cm^{-1};

^{13}C NMR, (300 MHz), solid state, δ (ppm): 26–37.4 (CH_2), 41.3 (CH_2), 61.7 (CH_2), 76.5 (C_q), 114.6 (weak) (=CH_2), 127–129.0 (CH_{Ar}), 135.0–140.0 (C_q), 145.6 (C_{Arq}), 157.3 (C=O), 179.9 (C=O).

The chemical structures of the obtained chlorinated hydantoin-containing polymers are shown in Figure 5 (where X=Cl).

4.12 Methods of characterization

^1H and ^{13}C NMR spectra were recorded at room temperature with Bruker AVANCE III 700- and 300-MHz spectrometers (Bruker, Germany). Chemical shifts (in parts per million) were determined relative to tetramethylsilane.

The FTIR spectra were recorded with a Mattson Genesis II spectrophotometer (range 700–3800 cm^{-1}; Mattson Instruments, WI, USA) in HCBD suspension.

The average molecular weights and polydispersity of *p*-chloromethylated polystyrene were determined using a high-performance liquid chromatographer coupled to a refractive index detector (Shodex Ri-71, Japan). The polymer was diluted to a final concentration of 3 mg/ml, and 125 µl was injected into the TSKgel GMHHR-M(S) (7.8 mm, ID 30 cm, 5 µm) size exclusion column (TosoHaas, Japan). Tetrahydrofuran was used as a mobile phase. The flow rate was 1 ml/min. The detector and column temperature was kept constant at 25°C. A standard calibration curve was prepared to determine the molecular weight of the fraction using 10 polystyrene standards (Sigma Aldrich, Saint Louis, MO, USA) with different molecular weights (from 1820 to 1.044×10^6 Da). The calibration curve was plotted as a logarithm of the molecular weight (log MW) as a function of retention time (t_r). The equation obtained from the standard curve of the molecular weight was as follows: $\log(M_p)=38.9042-5.9186t_r+0.3602t_r^2-7.8379\times10-3t_r^3$ with $r^2=0.9995$.

The thermal properties of the obtained hydantoins and polymers have just been published in a separate work (36).

4.13 Biocidal efficacy testing

Each *N*-halamine polymer was tested for biocidal activity against two strains of bacteria: a Gram-negative bacterium *E. coli* (ATCC 10536) and a Gram-positive bacterium *S. aureus* (ATCC 6538).

4.14 Bacterial suspension preparation

Isolated testing cultures were streaked on the slants of a nutrient agar [composition of the medium (g/l): bacteriological peptone 5.0, beef extract 3.0, and agar 15.0] and incubated overnight at 37°C.

The next day, 5 ml of sterile phosphate buffered saline (PBS) [composition (g/l): NaCl 8.0, KCl 0.2, Na_2HPO_4 1.44, and KH_2PO_4 0.24] was added to test tubes with the biomass of the bacteria and vortexed at 3000 rpm/min. A total of 0.5 ml of each strain suspension of the bacterium was taken and moved to a sterile test tube. Then, an appropriate volume of PBS was added, to obtain a suspension of cells with an optical density of about 0.8, according to the McFarland's scale. The required optical density was monitored by a densitometer at a wavelength of 600 nm.

The number of living and viable cells in the prepared suspension [N] was determined by the pour plate technique in triplicate, using R_2 agar (medium) with the following composition (g/l): bacteriological peptone 0.75, yeast extract 0.50, trypton 0.25, glucose 0.50, starch 0.50, sodium pyruvate 0.30, K_2PO_4 0.30, $MgSO_4$ 0.024, and agar 15.00. Bacterial colonies were incubated at 37°C for 48 h. After this time, the colony units were counted by multiplying the number of colonies by the dilution factor:

$$N=a\times D \tag{1}$$

where a is the average number of grown colonies on the plate and d is the dilution of bacterial suspension.

4.15 *N*-Halamine polymers and working suspension preparation

Each tested and reference substance (which was polystyrene) was weighed and suspended into appropriate volumes of sterile PBS. One milliliter of a previously prepared suspension of the test strain was added to 9 ml of prepared mixtures of tested substance, and it was shaken for a specified contact time, i.e., 5, 15, and 60 min (Table 6). The same procedure was also performed in the case of the corresponding referential sample. The five identical solutions of each specimen were prepared for repetition, and the results were averaged.

4.16 Determination of the number of living and viable cells after contact time with the tested substance [N_a]

After each contact time of the bacteria with the tested substance, the number of living and viable cells was determined by the pour plate technique in three repetitions. R_2 agar, which consists of tween 80 (30.0 g/l), lecithin 3.0 g/l, and L-histidin 1.0 g/l, was applied as a neutralizer. An analogous procedure was performed in the case of the reference suspension.

4.17 Experimental conditions of the control sample (validation of the experimental conditions and verification of the absence of any lethal effect in the test conditions)

For the determination of the survivability of the tested strains of the bacteria during the performed procedure, the referential substance was used. The prepared suspension of polystyrene powder with the same concentrations as those of the tested substances was studied using the same procedure as that for *N*-halamine polymers. Results of the validation confirmed the lack of influence of the performed procedure on the survivability of bacterial cells in the tested conditions.

4.18 Reduction in the number of living and viable cells of the tested bacteria [*R*]

The reduction in the number of cells capable of growth was calculated with the following formula (EN 1040:2005):

$$R=\frac{N\times 10^{-1}}{N_a} \tag{2}$$

where N is the number of living and viable cells of the bacteria in the tested suspension, 10^{-1} is the conversion factor concerning the dilutions of bacterial suspension, and N_a is the number of living and viable cells after contact with the tested substance during a chosen time.

Table 6 Composition of the tested suspensions with *N*-halamine polymers.

Sample weight (mg)	Volume of added PBS (ml)	Volume of suspension of the test strain (ml)	Final volume of solution (ml)	Final concentration of the solution (mg/ml)
5.0	9	1	10	0.5
10.0	9	1	10	1.0
50.0	9	1	10	5.0
100.0	9	1	10	10.0
250.0	9	1	10	25.0

4.19 Concentration of chlorine in water extracts

The chlorinated polymers, formerly purified, were immersed in water for 30 min at room temperature. After sampling the aliquot of the suspension, free chlorine was immediately analyzed spectrophotometrically (by colorimetric test). Free chlorine is that portion of chlorine present in extract in the form of dissolved elementary chlorine (Cl_2), as hypochlorous acid (HClO) and as hypochlorite ion (ClO^-). The method is based on the reaction of dipropyl-*p*-phenylenediamine (DPD), which forms a color semiquinoid dye with Cl_2 in weak acidic solutions (37). Ten milliliters of water extract was pipetted into the test tube and vigorously shaken with DPD reagent and left to stand for 1 min. A pH of 5.0 was kept. The absorbance at 557 nm of the formed red violet was measured using a Merck SQ118 photometer (Merck, Germany). Distilled water was used as a blank sample. The reagents were purchased from Merck (Spectroquant 100599). The test was also carried out for filtered extracts after a biocidal test, in which the polymers were in direct contact with bacteria (*E. coli* or *S. aureus*) in solution with two different density values (0.4 and 0.8) of the bacteria suspension. In this case, the chlorinated polymers were immersed in the suspension of bacteria for 15 min at room temperature. After this time, the polymers and bacteria were removed by filtration. For each sample, the measurement was repeated, and the results were averaged. The applied method is based on standards approved for drinking water and wastewater (EPA 330.5; EN ISO 7393).

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