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Polymeric iodophors with poly(2-ethyl-2-oxazoline) and poly (N-vinylpyrrolidone): optical, hydrodynamic, thermodynamic, and antimicrobial properties

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ABSTRACT

Formation of iodophors by interactions of poly(2-ethyl-2-oxazoline) and poly(N-vinylpyrrolidone) with molecular iodine dissolved in aqueous solutions with addition of potassium iodide and ethanol has been studied using a range of physicochemical techniques including UV–Vis spectroscopy, viscometry, dynamic light scattering, isothermal titration calorimetry and partitioning through semi-permeable membrane. It was established that poly(2-ethyl-2-oxazoline) exhibits greater ability to bind iodine compared to poly(N-vinylpyrrolidone). Despite the difference in the binding ability of these two polymers, their iodophors exhibited similar antimicrobial properties.

1. Introduction

The discovery of antiseptic properties of molecular iodine by Casimir Joseph Davaine in 1873 [1] has revolutionized many surgical procedures and this unique ability of iodine is still widely used in medical field. It is well established that iodine exhibits bactericidal, fungicidal, virucidal, and sporicidal activities [2]. Its aqueous solutions have been used as antiseptic to the skin and mucosal surfaces [3] and widely applied in wound treatment [4]. The mechanism for antimicrobial activity of iodine is very different compared to antibiotics, which reduces the potential for antibiotic resistance [5].

Iodine is a typical σ-acceptor and has the ability to participate in charge transfer interactions and form complexes with some polymers, having heterocyclic, aliphatic, and aromatic groups. These complexes are named iodophors and this complex formation often enhances the solubility of iodine in water, reduces some disadvantages of its aqueous solutions (irritation, toxicity, absence of odour) and improves disinfecting properties [6,7].

One of the first interactions of iodine with polymers was iodine–-starch reaction discovered in 1814 [8,9]. This reaction results in formation of intensely blue-black complex that has important applications in analytical chemistry [10]. Later, similar reactions of iodine were also discovered for some synthetic water-soluble polymers such as poly (vinyl alcohol) [11,12] and poly(N-vinylpyrrolidone) [13]. These iodophors have found wide applications as antiseptics in medicine. There are also reports about iodine complexes with chitosan [14], poly (ethylene glycol) [15] and cyclodextrins [16].

The complex of poly(N-vinylpyrrolidone) (PVP) with iodine, often called povidone-iodine, is the most widely used iodophor suitable for hand disinfection, skin preparation and antiseptic irrigation [17]. Povidone-iodine is on the World Health Organization's List of Essential Medicines [18] and it can be formulated as solutions, sprays, surgical scrubs, ointments, and swab dosage forms. The structural studies of povidone-iodine complex indicated that PVP units are protonated and linked together via hydrogen bonds and incorporate triiodide anions [19].

Poly(2-oxazolines) is an emerging class of polymers that have attracted substantial attention of researchers in the recent two decades [20–23]. Several nonionic poly(2-oxazolines) such as poly(2-methyl-2-oxazoline), poly(2-ethyl-2-oxazoline), poly(2-propyl-2-oxazoline and

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poly(2-isopropyl-2-oxazoline) are soluble in water. These polymers have excellent biocompatibility, cyto- and hemocompatibility [24], non-immunogenic character [21], and some of these exhibit stealth properties [25–28].

Poly(2-oxazolines) have some structural features resembling poly(N-vinylpyrrolidone), for example, presence of carbonyl group and amide bond, therefore these polymers are interesting as potential alternatives to PVP in many pharmaceutical and biomedical applications. For example, some recent studies demonstrated the use of poly(2-oxazolines) in the design of solid drug dispersions [29–31], tablets for oral drug delivery [32] and hydrophilic films for topical applications in combination with other water-soluble polymers [33]. However, to the best of our knowledge, currently there are no studies reporting the formation of iodophors based on poly(2-oxazolines) and it is not clear how iodine binding capacity of these polymers is compared to poly(N-vinylpyrrolidone) as the golden standard widely used commercially in this area.

In this work we used a range of classical and relatively new methods of physicochemical analysis to study formation of complexes of poly(2-ethyl-2-oxazoline) and poly(N-vinylpyrrolidone) with molecular iodine in aqueous solutions. These methods included UV–vis spectrometry, partitioning through semi-permeable membrane, viscometry, dynamic light scattering and isothermal titration calorimetry. Additionally, we evaluated antimicrobial properties of different iodophors formed.

2. Experimental section

2.1. Materials

A polyvinylpyrrolidone (PVP, $M_w \sim 44$–54 kDa and $M_w \sim 360$ kDa), poly(2-ethyl-2-oxazoline) (POZ, $M_w \sim 50$ kDa, PDI 3–4 and $M_w \sim 500$ kDa, PDI 3–4) and poly(acrylic acid) (PAA, $M_w \sim 450$ kDa) were purchased from Sigma-Aldrich. Potassium iodide, molecular iodine and other chemicals were of analytical grade and used without further purification.

2.2. Preparation of iodophors

Iodine solution was prepared by dissolving 1.6 g of molecular iodine in the presence of 12 g of potassium iodide (KI) and 40 mL of ethanol, then the total volume was made up to 1 L with deionized water (iodine concentration is 1.6 mg/mL) and stirred overnight at room temperature. After preparation, the solution was kept in the darkness. Iodophors were prepared by dissolving some amount of each polymer depending on the molar ratio of complex [polymer repeating unit]:[iodine] = 1:1, 5:1 and 10:1 during 1–2 h at room temperature. Formation of iodophors have been seen visually by changes in color of solutions.

2.3. Dynamic light scattering

The hydrodynamic radius of polymer-iodine complexes in solutions, R_h, was measured at a scattering angle of $\theta = 173^\circ$ using a Zetasizer Nano ZS instrument, model ZEN3600 (Malvern Instruments, UK). The DTS (Nano) program was used to evaluate the data. The z-averaged value of the apparent R_h was chosen to monitor the changes in the system as a function of iodine. Three measurements were recorded at each iodine concentration. Standard deviation for the R_h value was calculated using three measurements and was <5% for all samples. All solutions were filtered through a 0.45 μm polyvinyl difluoride syringe filter before measurement. Four solutions of PVP and POZ polymers were measured in iodine/KI/ethanol/water with the molar ratios [polymer repeating unit]:[iodine] = 1:1, 2:1, 5:1, and 10:1. The concentrations of PVP and POZ were fixed and set to 1.187 mg/mL and 1.105 mg/mL, respectively.

2.4. Isothermal titration calorimetry

The microcalorimetry study was performed using a MicroCal 200 isothermal titration calorimeter. The concentration of PVP used in these experiments was 0.156 mg/mL and the concentration of POZ was 0.198 mg/mL; the concentration of iodine was always 0.5 mg/mL. The solvent used to prepare these solutions composed of KI/water/ethanol mixture as described in section 2.2. The experiment was performed with consecutive injections of the iodine solution into the calorimeter cell; the cell contained 280 μL of the non-filtrated polymer solution or KI/water/ethanol. Iodine solution was added to a 40 μL injection syringe, the tip of which was modified to act as a stirrer. The chosen stirring speed was 750 rpm. The injection volume was 2 μL. The time between injections was 120 s. The measurements were performed at 25 °C. The data were analyzed using Microcal Origin and NITPIC [34,35] software. Experimental enthalpy was determined by integrating the raw data signal, and the integrated molar enthalpy change per injection was obtained by dividing the experimentally measured enthalpy by the number of moles of the iodine added. The final enthalpograms are the plots of the integrated molar enthalpy as a function of the total molar iodine concentration to polymer concentration in the calorimeter sample cell.

2.5. Partitioning through semi-permeable membrane

Partitioning of molecular iodine between the solution containing the polymers and polymer-free solution was studied using horizontal Ussing chamber, separated by semipermeable cellulose membrane (molecular weight cut off = 14 kDa; Sigma Aldrich).

The first chamber was filled with polymer in iodine/KI/ethanol/water solution with the molar ratio [polymer repeating unit]:[iodine] = 1:1, whereas the second chamber filled with solution of KI/ethanol/water (Fig. 1S). Ussing chamber was continuously shaken at 50 rpm/min during 48 h in the incubator at 25–26 °C. The amount of iodine in the second chamber was determined spectrophotometrically at 352 nm. The calibration curve was plotted using the iodine concentrations ranging within 0–0.025 mg/mL (Fig. 2S). For preparation of this calibration curve iodine stock solution was diluted with freshly prepared KI solution (1.2 g KI, 4 mL of ethanol in 100 mL of water). The total concentration of iodine in that system was 1.6 mg/mL, so the iodine concentration in the first chamber was calculated as the difference $[\text{iodine}]_{\text{(first chamber)}} = 1.6\text{-}[\text{iodine}]_{\text{second chamber}}$. The value of the partition coefficient was calculated as the ratio of iodine concentrations in the first chamber to the concentration of iodine in the second chamber. All measurements were carried out in triplicates and mean values ± standard deviations were calculated.

2.6. Viscometric analysis

Determination of intrinsic viscosity of polymer solutions in water, KI/ethanol/water and I_2/KI/ethanol/water was conducted using microviscometer Anton Paar with capillary having diameter 1.59 mm at 25 °C by measuring the efflux time of solvent (τ_0) and polymer solutions (τ). For measurement of viscosity five solutions were prepared with concentrations 1 g/dL, 0.75 g/dL, 0.5 g/dL, 0.375 g/dL and 0.1 g/dL in each of 3 solvents. All measurements were conducted in triplicates. The relative (η_{rel}) (1), specific (η_{spec}) (2) and reduced (η_{red}) (3) viscosities were calculated according to the following equations:

$$\eta_{rel} = t/t_0 \quad (1)$$

$$\eta_{spec} = t/t_0 - 1 = \eta_{rel} - 1 \quad (2)$$

$$\eta_{red} = \eta_{spec}/c \quad (3)$$

The values of intrinsic viscosity were determined by extrapolation of η_{red} values obtained at several concentrations to infinite dilution using

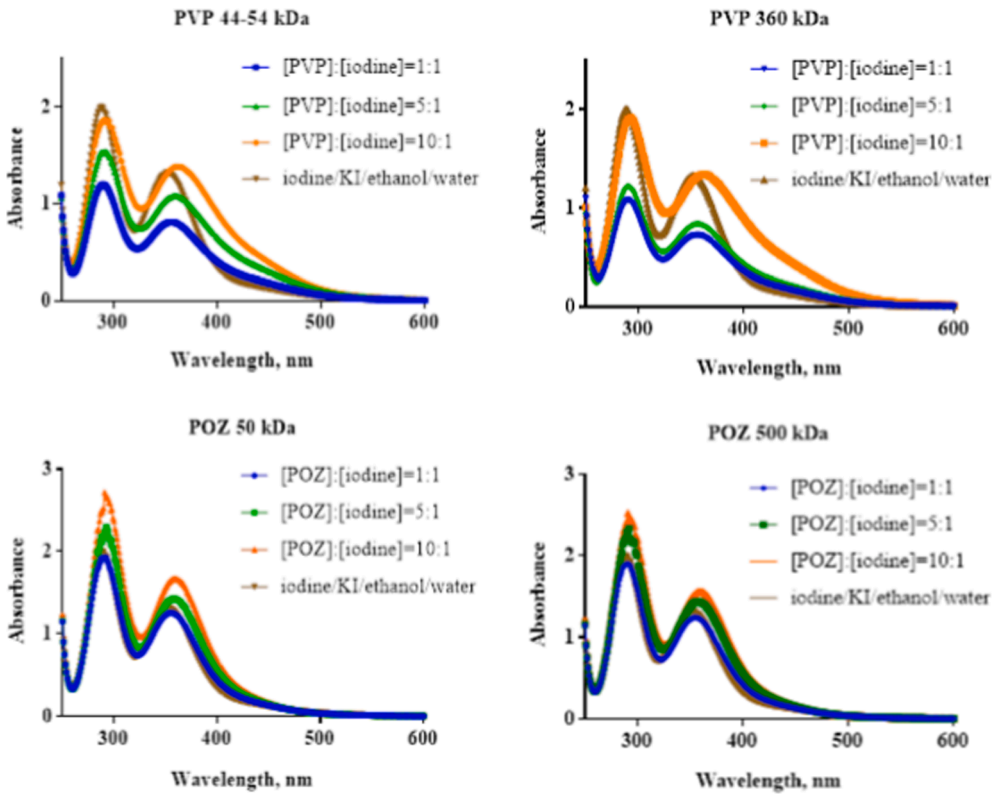


Fig. 1. UV–vis spectra of PVP-iodine and POZ-iodine solutions with molar ratio [polymer repeating unit]:[iodine] = 1:1, 5:1, 10:1 and 0.04 mg/mL iodine solution.

Fig. 2. Solutions of iodine (1) and their complexes with 50 kDa POZ (2) and 44–54 kDa PVP (3). Concentration of iodine in these solutions was 0.004 w/v % with 1.2 w/v % KI. Concentrations of POZ and PVP were 0.0078 w/v % and 0.0175 w/v %, respectively.

Huggins equation [36,37].

$$\eta_{red} = [\eta] + k_H[\eta]^2 c; [\eta] = \lim_{c \to 0} \eta_{red}$$

where k_H is the Huggins constant.

Concentration dependences of the reduced viscosity for polymer solutions were approximated by linear law and are provided in supplementary information (Figure S3). Huggins constants were determined from the slope of the linear fit.

2.7. UV–visible spectrophotometric analysis

UV–vis absorption spectra of iodine and polymer-iodine complexes were recorded using UV-spectrophotometer «Analytik Jena» (Japan) with a quartz cuvette of 1 cm thickness at room temperature with deionized water as a reference.

2.8. Studies of antimicrobial properties

Antimicrobial properties of polymer iodophors of PVP and POZ were studied using zone–of-inhibition disk diffusion assay (DDA) and serial dilutions method. *Staphylococcus aureus* ATCC 6538-P was obtained from the Republican Collection of Microorganisms (RCM, Nur-Sultan, Kazakhstan) and *Candida albicans* ATCC 10,231 were received from the American Type Culture Collection (ATCC, USA).

DDA was carried out by applying the discs treated with the iodophors in Petri dishes with suspensions of microbial test-strains using sterile forceps at a distance of 15–20 mm from the edge of the dish and from each other. For analysis 3 solutions of each polymer were prepared in iodine/KI/ethanol/water with the molar ratios [polymer repeating unit]:[iodine] = 1:1, 5:1 and 10:1. Solution of pure iodine was applied as a control sample. Petri dishes were inoculated with a suspension of test strains with a density of 1.5×10^8 CFU/mL. For seeding, sterile cotton swabs were used, immersed in a suspension of a microorganism, and then streaked in three directions, turning the dish by 60°. For the study, cartridges with ready-made sterile discs (Himedia, India) were used. The disks were preliminarily saturated with the solution of iodophor; the exposure time was ≈30 min. The incubation time was 18–24 h at 37 °C for bacteria. The disk diffusion assay results were taken into account by calculating the diameter of the growth retardation/suppression zones with an accuracy of 1 mm. All experiments were conducted in triplicates.

A 48-well plate was used to determine antimicrobial activity by serial dilutions method. All wells were filled with 500 μL Mueller-Hinton

nutrient broth (for testing *Staphylococcus aureus*) and Sabouraud broth (for testing *Candida albicans*) from 1st to 8th well, the 9th well was used as a culture control. Iodine-polymer complexes were introduced in pure form (500 μL) into 1st well. Serial dilutions were carried out starting from 1st well by adding the mixture (nutrient broth (500 μL) + iodophor (500 μL)) from 1st tube in the amount of 500 μL into 2nd tube, already containing 500 μL of nutrient broth. The following dilutions were prepared: 1:1, 1:2; 1:4; 1:8; 1:16; 1:32; 1:64; 1:128, which corresponds to wells 1 to 8, and the 9th well served as a culture control. After dilutions, 50 μL of test strains of microorganisms were added to all wells at a concentration of 1.5×10^6 CFU/mL for *Staphylococcus aureus* and 7.5×10^6 CFU/mL for *Candida albicans*. All samples were incubated for 18–24 h at 37 ± 1 °C. After the incubation, plating was carried out in Petri dishes with Mueller-Hinton agar-agar to count CFUs.

2.9. Statistical analysis

The mean values ± standard deviation were compared for significance using two-tailed Student's *t*-test and a one way analysis of variance (ANOVA) with GraphPad Prism statistical analysis software (version 7.0; Graph Pad Software Inc.), where $p < 0.05$ was set as the statistical significance criterion.

3. Results and discussion

Iodine has a relatively poor solubility in water (~0.33 g/L); however, it dissolves well in ethanol and also its solubility in water is greatly improved in the presence of iodide ions due to the formation of water-soluble triiodide ions (I_3^-) and other ions [6]. Typically, a combination of potassium iodide (KI) and ethanol is used in commercial iodine formulations to achieve an optimal concentration of iodine in water. For this reason, aqueous solutions containing KI and ethanol were used in this study as a solvent for iodine, its mixtures and complexes with polymers. Since the solutions of iodine always consist of a complex mixture of molecular iodine and iodine-containing ions, in the paper we will refer to all species as simply iodine.

Aqueous solutions of iodine are yellowish-brown in colour and upon complexation with polymers the intensity and even the colour of solutions often change dramatically [6]. These changes in colour make the use of optical methods such as spectrophotometry to be highly valuable to study formation of iodophors. The UV–vis spectra of iodine, PVP-iodine and POZ-iodine in aqueous solutions show the presence of absorption peaks at ~ 290 and ~ 350–360 nm (Fig. 1), which are typical for aqueous solutions of iodine and iodophors as described in literature [6]. The absorbance maxima of these solutions at around 192–193 nm and 226 nm (this spectral region is not shown in Fig. 1) are known to be due to iodide ions I^- [6]. Historically, it was believed that both peaks at ~ 290 nm and ~ 350–360 nm belong to I_3^- ions [38]; however, later it was demonstrated that ~ 290 nm belongs to I_3^- and ~ 350–360 nm is due to IO^- oxyanion [39]. Additionally, solvated molecules of I_2 were also reported to show a maximum at 450–460 nm [39]. Our spectral data show that addition of PVP to solutions of iodine in KI/ethanol/water mixture results in lowering of the absorbance at ~ 290 and ~ 350–360 nm at [PVP]/[iodine] = 1:1; however, a further increase in [PVP]/[iodine] to 5:1 and 10:1 leads to increase in the intensity of these peaks. Clearly these spectral shifts indicate some changes in I_3^- and IO^- concentrations related to the complexation with PVP. There is also a clear bathochromic (red) shift from 290 nm to 292–293 nm and from 350 nm to 353–360 nm, which makes these solutions more intensively colored. The appearance of absorbance in 400–500 nm region could be associated with the increase in the concentration of solvated molecules of I_2 (however, a characteristic maximum at 450–460 nm is not observed). These changes are observed for PVP of both molecular weights (44–54 kDa and 360 kDa).

Spectral changes are also observed upon mixing of iodine with POZ. However, unlike the case with PVP, the increase in [polymer repeating unit]:[iodine] molar ratio leads to increase in the absorbance in solution mixtures of POZ with iodine. Additionally, a bathochromic shift is also observed for complexation of POZ with iodine. However, unlike for the complexes with PVP, the substantial increase in absorbance in 400–500 nm region is not observed. As in the case with PVP, there is no substantial difference in the UV–vis spectra recorded for iodophors formed by POZ with 50 and 500 kDa. The changes happening with iodine upon its complexation with both polymers are clearly visible visually (Fig. 2). Overall, these results indicate that both PVP and POZ do form complexes with iodine, which results in some changes in their UV–vis spectra. However, there is a clear difference in the spectral characteristics observed for the complexes of iodine with POZ compared to PVP.

The ability of polymers to bind iodine and form complexes can be evaluated using a diffusion cell consisting of two equal-volume compartments separated with a semi-permeable dialysis membrane. If a solution of iodine or iodophors in KI/ethanol/water are placed into one compartment (donor chamber) and the second compartment (receptor chamber) is filled with the solvent (KI/ethanol/water) then unbound iodine will partition between two cells. The degree of this partitioning will depend on the binding ability of the polymers and can be easily estimated using a partition coefficient (PC) using the following equation:

$$PC = [iodine]_{(donor\ \ chamber)} / [iodine]_{(receptor\ \ chamber)} \quad (5)$$

The partition coefficient (PC) characterizes the equilibrium between the concentrations of iodine in two compartments and depends on the nature of the interacting system and temperature. PC proposed in this work is to some extent similar to the partition coefficient (LogP) that is commonly used in pharmaceutical sciences to characterise partitioning of drug molecules between water and n-octanol [40].

In our experiments, the starting concentration of iodine in the donor chamber was always kept at 1.6 mg/mL and in the receptor chamber initially it was 0 mg/mL. After 48 h of equilibration of the diffusion cell the iodine partitioned between two chambers and its concentration was determined spectrophotometrically. Additionally two control experiments were conducted when iodine was left to partition from polymer free solutions and also from solutions of poly(acrylic acid) (PAA). PAA was chosen as an anionic polymer that was assumed to be unable to form complexes with iodine. The experimental results on determination of PC values are presented in Fig. 3. In the case of iodine partitioning from polymer-free and PAA-iodine solutions the partition coefficients were about 1. This confirmed our hypothesis about the absence of iodine

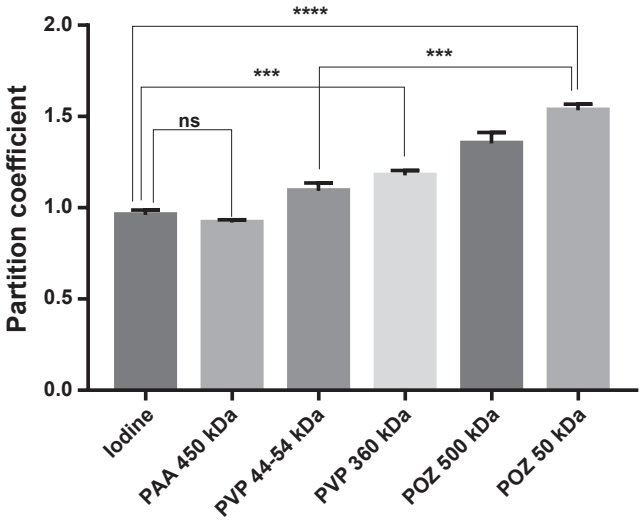


Fig. 3. Partition coefficients for pure iodine and its mixtures with PAA 450 kDa, PVP 44–54 kDa, PVP 360 kDa, POZ 50 and 500 kDa. All values are the means ± standard deviations of triplicate experiments. Statistically significant differences are given as: *** - $p < 0.001$; ** - $p < 0.01$; * - $p < 0.05$.

binding by PAA. For all PVP-iodine and POZ-iodine mixtures the partition coefficients were always>1, indicating retention of iodine in the donor chamber associated with its specific binding by the polymers. The values of PC determined for different polymer samples can be arranged in the following order: POZ (50 kDa) = POZ (500 kDa) > PVP (360 kDa) = PVP (44–54 kDa). There was no statistically significant difference ($p > 0.05$) between PC values of the same polymer but different molecular weight. However, POZ clearly exhibited greater PC values compared to PVP samples, indicating stronger ability of POZ to bind iodine.

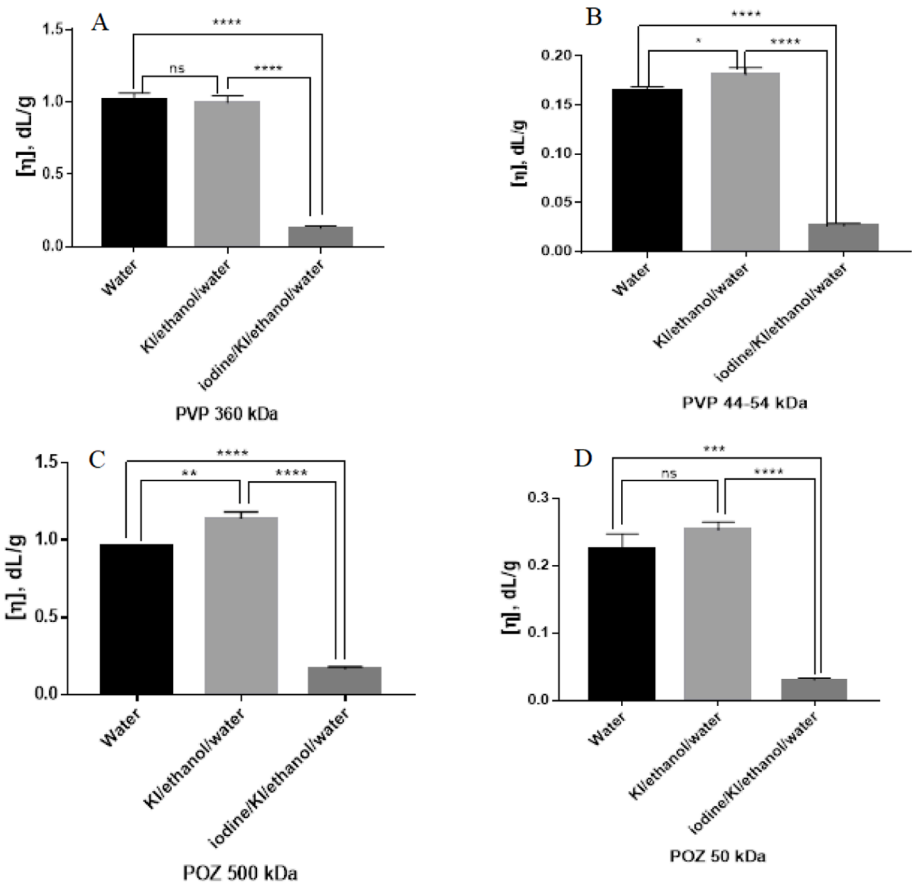
Having hypothesized the iodine complex formation for POZ, we used viscometry to study the hydrodynamic behavior and conformation of polymer chains in solution. The conformation of macromolecules in solution is the tradeoff of several interactions such us polymer–solvent, solvent–solvent, and polymer–polymer that participate in enthalpic and entropic contributions to Gibbs free energy [41,42]. In a thermodynamically good solvent, the polymer–solvent interactions prevail over polymer–polymer ones, resulting in an expansion of the chain; while in a thermodynamically poor solvent, the polymer–polymer interactions dominate, resulting in contraction of the chain. Apart from enthalpy driven conformation changes, entropy contribution is of paramount importance for thermoresponsive polymers such as poly(N-isopropylacrylamide), poly(vinyl methyl ether) and poly(2-ethyl-2-oxazoline) [43,44]. At room temperature, PVP and POZ are soluble in water and potassium iodide/ethanol/water mixture that implies good thermodynamic quality of these solvents. This conclusion is supported by a number of reports providing experimental evidence that PVP and POZ have an expanded coil conformation in aqueous solutions [45,46]. Both polymers have similar equilibrium rigidity of 1.7–1.8 nm that attributes them to the class of flexible polymers. The choice of viscometry as a tool to monitor the complex formation stems from high sensitivity of intrinsic viscosity [η] to any particle density alterations caused by conformational changes. Indeed, $[\eta] \sim \frac{R_g^3}{M_w} = \frac{1}{d}$, meaning that intrinsic viscosity value is inversely proportional to particle's density [47]. Complex formation is usually followed by the particle density increase manifested as a decrease in intrinsic viscosity value. As it can be seen from Fig. 4**(A–D)** and Table 1, the value of intrinsic viscosity for PVP 360 kDa and PVP 44–54 kDa in water demonstrates a similar profile with solution in KI/ethanol/water ($p < 0.05$ and $p>0.05$, respectively). Whereas in the solution of iodine/potassium iodide/ethanol/water we observed a sharp decrease in intrinsic viscosity ($p < 0.0001$) compared to the values in water and KI/ethanol/water (Table 1). These results are in agreement with the report by Kirsh et al [48], who previously reported a drop in viscosity upon complexation of PVP with iodine. Such a drop is another evidence of the polymers complexation with iodine leading to the contraction of the polymer chains. As can be seen from Fig. 4, the values of intrinsic viscosities in water and potassium iodide/ethanol/water solution for POZ 50 kDa and 500 kDa differ slightly (0.23 vs 0.26 ($p>0.05$) and 0.96 vs 1.01 ($p < 0.01$, respectively), which eliminates the possible influence of ionic strength. However, intrinsic viscosity in the solution of potassium iodide/ethanol/water and iodine shows significantly lower values ($p < 0.01$) compared to two other values for these polymers.

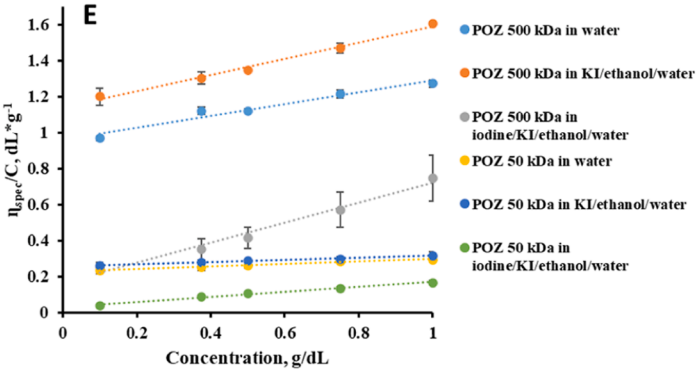
Although POZ and PVP show very close values for intrinsic viscosity in the same solvent, their Huggins constants reveal new insights on the difference in polymer conformation. In general, Huggins constant values for PVP are 40–70% higher in comparison with POZ of the similar molecular weight. Indeed, k_H value of PVP 360 kDa is 0.6 ± 0.2, whereas POZ 500 kDa has a much lower value (0.37 ± 0.03) in water. Similar trend was observed for POZ 50 kDa and PVP 44–54 kDa in water and KI/ethanol/water solvents. The values of the Huggins constant could be used to assess the polymer conformation since it is extremely sensitive to the macromolecule − solvent interactions. k_H values can vary from 0.3 to 0.8 in thermodynamically good solvents with a general tendency to increase for thermodynamically poorer solvents. Thus, we concludethat water and KI/ethanol/water have better thermodynamic quality for POZ in comparison with PVP. We can expect the enhanced compaction of PVP macromolecules in these particular solvents with the decrease in PVP polymerization degree. The k_H values for PVP 44–54 kDa of 1.7 ± 0.7 and 1.43 ± 0.07 for water and KI/ethanol/water, respectively, are in line with this hypothesis indicating compact macromolecular conformations in solution. The presence of iodine results in significant deterioration of the thermodynamic solvent quality as manifested by high values of k_H (Table 1). Due to the very low values of intrinsic viscosity, the experimental error for k_H of iodophors is very high and we cannot make any additional judgments concerning the conformation of PVP and POZ in complexes.

Given the intrinsic viscosity results, we would expect 2–3-fold changes of polymer sizes upon complex formation. Dynamic light scattering experiments were performed to prove this conclusion. In general, the distribution functions show a single mode that was attributed to the diffusion of a single polymeric chain. At low ratios, the size of PVP and POZ macromolecules, undoubtedly decreases with increasing concentration of iodine in solution in line with viscometry data (Fig. 5). Z-averaged hydrodynamic radius value drops from 7.6 to 4.8 nm with iodine content increasing from 0 to 0.5 M ratio for POZ. With the dependence of intrinsic viscosity as third power of gyration radius, [η] value should decrease 4 times that corroborates with viscometry findings (Fig. 4). Similar trend was observed for PVP, where R_h value also decreases at low iodine concentrations although the drop is not pronounced. Surprisingly, the R_h dependence as a function of [Iodine]:[polymer repeating unit] ratio exhibits a clear inflection point. The inflection point depends on the polymer nature: 0.5 and 0.2 for POZ and PVP, respectively. The distribution R_h function unequivocally indicates the formation of sub-micron particles at molar ratio above 0.5 for PVP. Their formation is evident without DLS experiment; 0.45 μm PVDF filter was easily clogged during the filtration of PVP solution with iodine at 1:1 ratio. In contrast, POZ shows a very mild increase of R_h at high molar ratios. We can speculate that at this point POZ has higher binding affinity in comparison with PVP. At low molar ratios, iodine binds to a polymer forming unimolecular complex. At ratio 0.5, meaning one iodine molecule or ion per two repeating units, the unimolecular binding stops due to reaching a saturation point; all repeating units are bound with iodine. Previous reports on iodophors formation also suggested 1:2 stoichiometry ratio for PVP [6]. Further addition of iodine results in unimolecular complexes aggregation. We presume that for PVP the intermolecular aggregation starts at lower concentration of iodine due to lower affinity between iodine and PVP repeating units in comparison with POZ in line with the difference in k_H values obtained from viscometry experiments. POZ binding in turn appears to be much stronger since the onset of aggregation begins at higher iodine concentration.

Isothermal titration calorimetry (ITC) experiments were performed to prove this working hypothesis. ITC is a golden standard to explore various biological and soft matter systems including protein–ligand interactions [49], micellization-demicellization [50,51], pharmaceuticals [52–54], polymer-surfactant interactions [51,55–58], and polymer–polymer complex formation [59,60].

The titration of iodine to PVP and POZ isotherms show a clear biphasic behavior. The heat of injection has obvious trend to increasing the negative enthalpy at low [iodine]:[polymer repeating unit] ratios (Fig. 6). However, at higher values there is an opposite trend with steep decrease in negative enthalpy followed by a mild growth with the tendency to reach a plateau. Several features should be noted here. First, there is no obvious merging of the titration curves with the blank experiment even at 1:1 stoichiometry. A lack of merging is a direct manifestation of additional thermodynamic processes existing in iodophors formation apart from the classical $A + B \leftrightarrow AB$ hetero association model. The second issue is the striking difference in local minimum of the isotherms for PVP and POZ. The existence of isotherm's biphasic or polyphasic structure is not something new. Usually, such feature is reported for proteins with two or more non-equivalent binding sites [61].





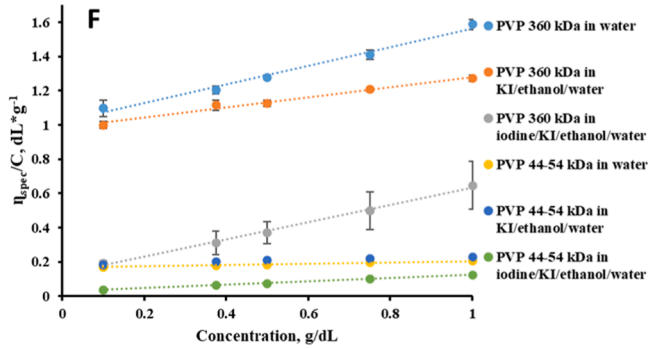


Fig. 4. (A-D): Intrinsic viscosity values in three different solvents for four polymers. All values are the means ± standard deviations of triplicate experiments. Statistically significant differences are given as: **** - $p < 0.0001$; *** - $p < 0.001$; ** - $p < 0.01$; * - $p < 0.05$. (E) Concentration dependence of reduced viscosity for PVP, open symbols – PVP 360 kDa; closed symbols - POZ 44–54 kDa; (F) concentration dependence of reduced viscosity for POZ, open symbols - POZ 50 kDa; closed symbols - POZ 500 kDa.

Table 1
Comparative table of intrinsic viscosities ([η]), and Huggins constants (k_H) for different solvents.

	water		KI/ethanol/water		Iodine/KI/ethanol/water	
	[η], cm^3/g	k_H	[η], cm^3/g	k_H	[η], cm^3/g	k_H
POZ 500	0.96 ± 0.01	0.4	1.10 ± 0.01	0.42 ± 0.01	0.13 ± 0.06	50.0
POZ 50	0.23 ± 0.03	0.8	0.26 ± 0.01	1.00 ± 0.50	0.04 ± 0.01	44.0
PVP 360	1.00 ± 0.07	0.6	0.98 ± 0.01	0.32 ± 0.01	0.14 ± 0.01	22.0
PVP 44–54	0.16 ± 0.01	1.7	0.18 ± 0.01	1.43 ± 0.07	0.02 ± 0.01	>100

Such isotherms were successfully fitted with "two binding sites" or sequential binding sites" models implemented in some widely used ITC-fitting software [62]. However, polyphasic behavior was also evidenced in some more sophisticated systems [63]. The analysis of such isotherms is far from trivial. Taking into consideration the polymers structure, a series of identical repeating units, the concept of two unique binding sites should be discarded. Previously, we reported on coiled coil peptides and polymer–peptide conjugates self-assembly with biphasic nature of isotherms [59].

The dissociation of homodimers was assumed as the driving force of the first exothermic process at low molar ratios. Taking into consideration these findings, an obvious question arises: does the first process come from any kind of disassembling or dissociation? We additionally inspected the freshly prepared non-filtrated solutions of PVP and POZ by DLS. The distribution functions reveal an additional small peak of few hundred nanometers that was attributed to a negligible fraction of polymer aggregates. The solutions were filtered and titrated the same day with iodine using ITC method as described in Experimental section. The titration isotherms for filtered and non-filtered solutions were identical within experimental error. Thus, we have to eliminate the dissociation mechanism and focus on other possible reasons. The most natural source of the first phase is, in our opinion, polymer conformational transition. Indeed, the complex formation should restrict the conformational mobility of polymeric chains. This entropic contribution will be seen as accompanying to the major binding event – the binding of iodine with polymer repeating units and will be pronounced at the earliest injections since the conformational changes will be the strongest. This assumption corroborates with DLS results, where the decrease in the polymer size was observed at low [iodine]:[polymer repeating unit] ratios. The second phase was attributed to iodine binding event. We have fitted the second phase of the isotherms with "one-site" binding model implemented in Microcal ITC software [64]. As shown in Fig. 6**A**, the second phase is enthalpy and entropy driven. Both contributions, ΔH and -TΔS, bring negative values to the Gibbs free energy change, ΔG, Fig. 6**B**. However, PVP shows greater values for enthalpy contribution and lower values for entropy one in comparison with POZ. In turn, the POZ binding constant is 30 %

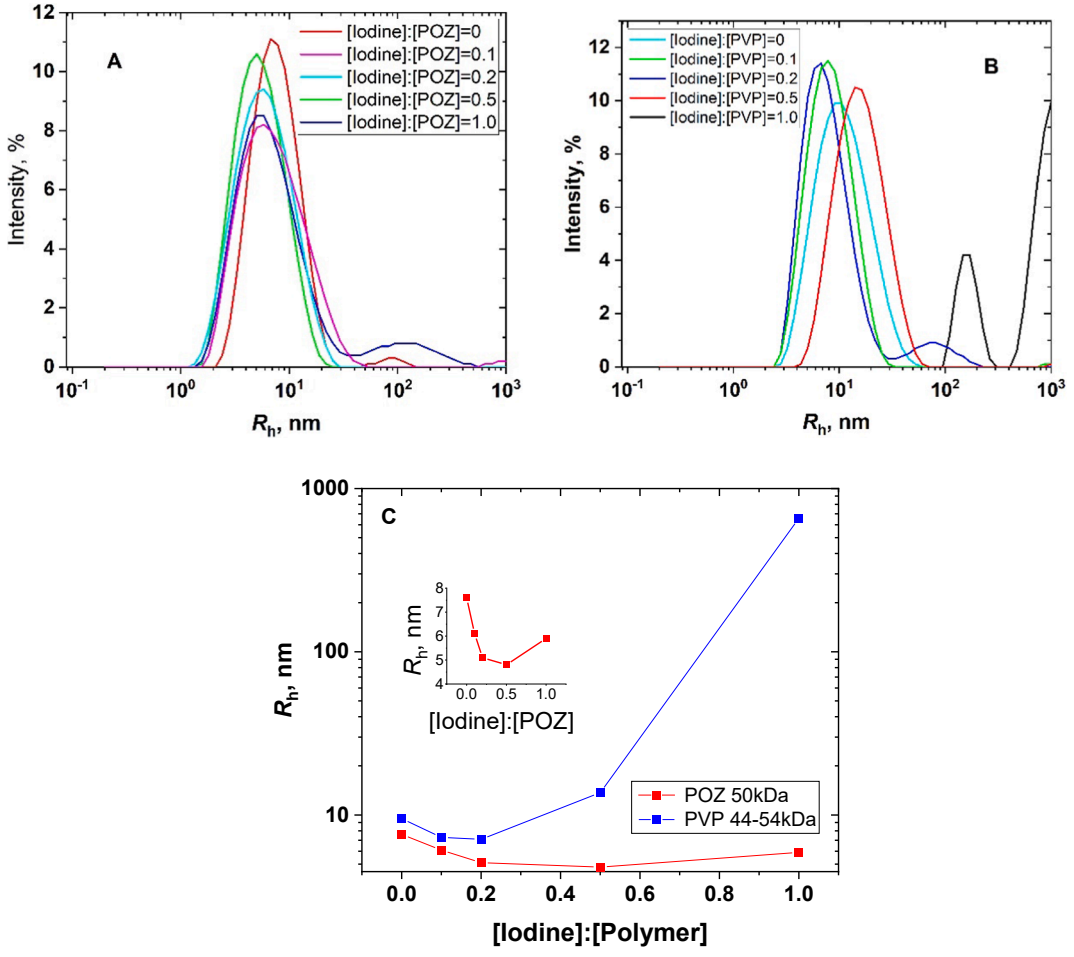


Fig. 5. A: The intensity-weighted distribution functions over hydrodynamic radius R_h for different [Iodine]:[POZ repeating unit] molar ratios; B: The intensity-weighted distribution functions over hydrodynamic radius R_h for different [Iodine]:[PVP repeating unit] molar ratios; C: The dependence of z-averaged value hydrodynamic radius R_h as a function of [Iodine]:[polymer repeating unit] ratio. Inset: z-averaged value of hydrodynamic radius R_h as a function of [Iodine]:[POZ repeating unit] ratio.

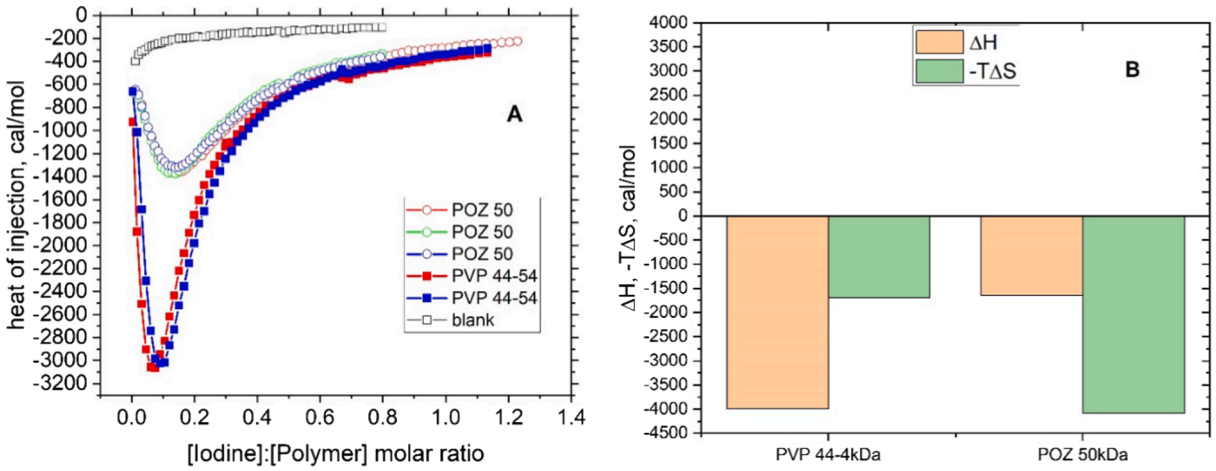


Fig. 6. A: Isothermal calorimetric titration of iodine solution in KI/ethanol/water solvent to polymer solution in the same solvent at T = 25 °C. Blank experiment with titration of iodine to KI/ethanol/water solvent is also depicted on the graph (open black squares). B: Entropy and entropy contributions as the fitting result of the isotherm second phase.

higher. Additionally, the binding stoichiometry is two-fold higher, 0.16 vs 0.39, for POZ. This is, to our best knowledge, the first report on greater iodine binding affinity and stoichiometry for POZ in comparison with classical iodophor forming PVP. The difference in binding stoichiometry justifies the difference in inflection points in DLS data. The presence of a long asymptotic behaviour is another riddle that should be addressed. We are inclined to associate this asymptotic process to iodophors aggregate formation observed by DLS. Thus, the iodine-to-polymer binding scenario looks as follows: first iodine molecules/ions bind to several repeating units significantly restricting polymer conformational mobility. These changes are visible on ITC thermograms as phase I characterized by increasing negative enthalpy. Originally flexible polymers adopt contracted frozen conformation with the onset of phase II. The phase II, in turn, is governed by "one-site" binding event between iodine and polymer repeating units. It is highly likely that iodine binds to two monomeric units of POZ. This could be either on the same chain or different chains. Having all POZ repeating units bound, the iodophors aggregation commences as the final step.

Previously, Schenck and co-workers [65] have published infrared spectroscopy and X-ray study reporting the mechanism of complexation and structure of PVP-iodine complexes. They proposed that in this complex, a proton is bound via hydrogen bonds with carbonyl oxygen atoms of two pyrrolidone rings. The triiodide anion is coordinated ionically to this proton. It is likely that iodine interacts with POZ in a similar manner; however, further structural studies will be required to establish the exact mechanism of this interaction.

It was interesting to elucidate whether the difference in the ability of polymers to bind iodine will have some effects on the antimicrobial properties of iodophors. In this study we have compared antimicrobial activity of iodophors formed by two polymers (PVP and POZ) against Staphylococcus aureus and Candida albicans.

Povidone-iodine is known to have very high efficiency against the most common organisms, including Staphylococcus aureus and Candida albicans [6]. In this work we have evaluated antimicrobial properties of all the studied iodophors using zone–of-inhibition disk diffusion and serial dilutions methods. Fig. 7 shows the values of zones of

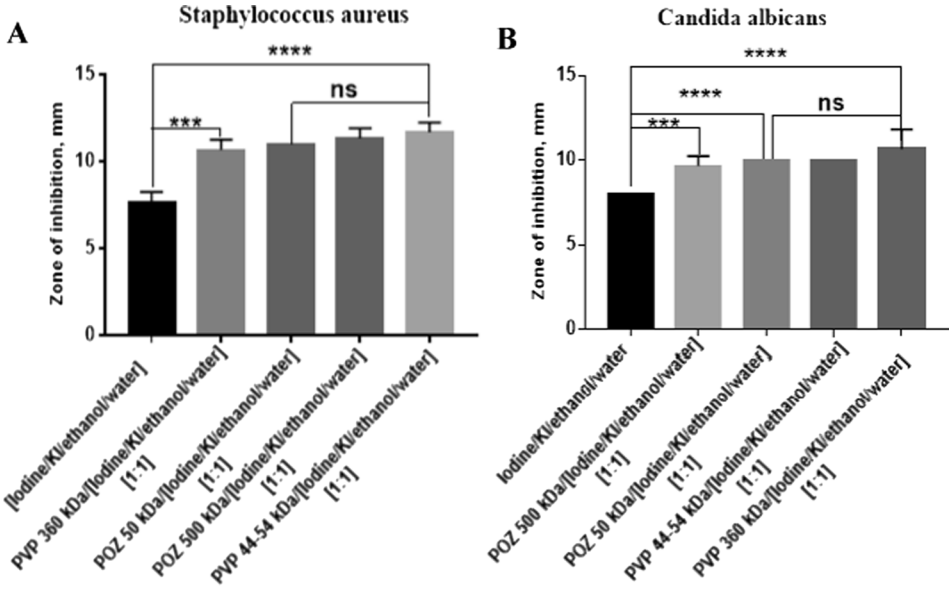


Fig. 7. Comparison histograms of zone of inhibition values (mm) for four polymers against Staphylococcus aureus (A) and Candida albicans (B). All values are the means ± standard deviations of triplicate experiments. Error bars are not present in those results where standard deviation was very close to zero. Statistically significant differences are given as: **** - $p < 0.0001$; *** - $p < 0.001$.

microorganism inhibition in the presence of free iodine solutions and different iodophors. Fig. 4**s** shows additional antimicrobial data for other polymer/iodine combinations. All iodophors exhibit greater zones of inhibition compared to polymer-free iodine. In addition, with the growth of polymer/iodine molar ratio in the complex zone of inhibition slightly decreases. It is related to decreasing concentration of available bactericidal free iodine with increase in polymer to iodine ratio. Iodophors based on PVP and POZ have similar values of zone inhibition against Staphylococcus aureus and Candida albicans. There are no significant differences between all polymers ($p>0.05$). The results of serial dilution method are summarized in Fig. 5S-7S. These do not show any difference in the antimicrobial activities between different iodophors. The minimal inhibitory concentration of iodine in all formulations was observed at 0.8 mg/mL.

It is well known that iodine is present in iodophor solutions in several states, which include I^-, I_2, I_3^- and other forms [6,39]. Iodide (I^-) ions are nonoxidizing species and therefore do not have antimicrobial properties. Molecular iodine (I_2) is the only species with an established bactericidal activity and its solvated forms $I_2{\cdot}HO$ or $I_2{\cdot}C_2H_5OH$ are believed to be the real microbicidal agents in aqueous and alcoholic solutions [66]. Triiodide (I_3-) is believed to not exhibit antimicrobial activity; however, it is the main species responsible for staining of biological tissue. If the complexation mechanism of iodine occurring through ionic coordination of triiodide ions with carbonyl groups of polymers is true [65], then this explains why there is no difference between the antimicrobial activities of iodophors derived from different polymers (PVP and POZ) as I_3^- species do not exhibit germicidal activities [66]. Nevertheless, we believe that the relationship between the structure of iodophors and their antimicrobial activities will require further studies.

4. Conclusion

Formation of complexes of poly(2-ethyl-2-oxazoline) and poly(N-vinylpyrrolidone) with molecular iodine has been studied in aqueous solutions containing potassium iodide and ethanol using a series of physicochemical techniques, including UV–vis spectrophotometry, viscometry, dynamic light scattering, isothermal titration calorimetry and partitioning through semi-permeable membrane. It was established that poly(2-ethyl-2-oxazoline) does form complexes with iodine similarly to poly(N-vinylpyrrolidone) and this complexation results in more intensive solution colour, compaction of macromolecules and subsequent aggregation of the complexes. Poly(2-ethyl-2-oxazoline) exhibited greater ability to bind iodine compared to poly(N-vinylpyrrolidone). However, despite the differences in the binding of iodine by the two polymers, the iodophors on their basis exhibit similar antimicrobial activity.

CRediT authorship contribution statement

Danelya N. Makhayeva: Investigation, Methodology, Writing – original draft. **Sergey K. Filippov:** Investigation, Methodology, Funding acquisition, Writing – original draft, Writing – review & editing. **Sanzhar S. Yestemes:** Investigation. **Galiya S. Irmukhametova:** Funding acquisition, Supervision, Writing – review & editing. **Vitaliy V. Khutoryanskiy:** Conceptualization, Supervision, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.eurpolymj.2022.111005.

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