**ORIGINAL ARTICLE**

# EVALUATION OF ANTIBACTERIAL ACTIVITY OF TWO POLY-(CARBOXYBETAINES) DERIVED FROM POLY(4-VINYLPYRIDINE)

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**Abstract**

Polybetaines have been still intensively studied due to their biomedical potential. In this work, two poly(carboxybetaines) derived from poly(4-vinylpyridine) P4VP that have methylene (P4VPB-1) or ethylene (P4VPB-2) spacer between $N^+$ and $COO^-$ groups were preliminary investigated by viscometry in salted aqueous solutions in order to correlate the "anti-polyelectrolyte" behaviour and conformational state of these polymers with their antibacterial potential. The microbiological tests revealed that P4VPB-1 did not interfere with the metabolism of *Escherichia coli* ATCC 25922 or *Staphylococcus aureus* ATCC 25923. Also, P4VPB-2 inhibited only the growth and multiplication of *S. aureus* and recorded a minimum inhibitory concentration (MIC) of 6.4 mg/mL.

**Rezumat**

Polibetainele sunt în continuare studiate datorită potenţialului biomedical. În această lucrare, două poli(carboxibetaine) derivate de la poli(4-vinilpiridină), ce conţin un spaţiator metil (pentru P4VPB-1), respectiv etil (pentru P4VPB-2) între grupările $N^+$ şi $COO^-$, au fost evaluate preliminar prin vâscozimetrie în soluţii apoase saline pentru a corela comportarea de „antipolielectrolit" şi starea conformaţională a acestor polimeri cu potenţialul lor antibacterian. Testele microbiologice au relevat faptul că P4VPB-1 nu a interferat cu metabolismul bacteriilor *Escherichia coli* ATCC 25922 şi *Staphylococcus aureus* ATCC 25923. Totodată, P4VPB-2 a inhibat numai creşterea şi multiplicarea bacteriei *S. aureus* şi a înregistrat o concentraţie minimă inhibitorie (MIC) de 6,4 mg/mL.

**Keywords**: poly(carboxybetaine), antibacterial agent, Kirby-Bauer diffusion method, resazurin test

## Introduction

P4VP is an interesting polymer with remarkable properties, making it available for many applications. The chemical modification of this polymer with suitable low molecular compounds lead to macromolecular chains which contain both positive and negative charges in the same monomer unit, named polybetaines [1].

In commercial terms, the polybetaines are described by their ability to provide a macromolecular support for the preparation of various materials, such as: sorbents, oil recovery agents, fungicides, flame-retardant polymers, wetting agents, dyeing agents in textile industry and cryoprotectors [2, 3], as well as drug delivery systems or cosmetic formulations [4-8]. Other research groups were focused on the antimicrobial properties of 4-vinylpyridine derivatives. Park *et al.* [9] studied the copolymers of styrene with 4-vinyl pyridine quaternized with *n*-octyl iodide. Also Zhang *et al.* [10] grafted P4VP onto halloysitenanotubes, and then immobilized silver ions on P4VP.

In this paper, the solution behaviour of poly(carboxybetaines) P4VPB-1 and P4VPB-2 was studied in order to find an explanation for their interactions with representative bacteria (*E. coli* and *S. aureus*) in salted aqueous media.

## Materials and Methods

*Reagents*

P4VP ($\overline{M}_v$ = 60,000 g/mol), acrylic acid, sodium chloroacetate, sodium chloride and calcium chloride were purchased from Aldrich Chemical Co. The acrylic acid was distilled in vacuum prior to use.P4VPB-1 was obtained by a nucleophilic substitution reaction of P4VP with sodium chloroacetate, and P4VPB-2 from a nucleophilic addition reaction of acrylic acid to P4VP. The polymerization of P4VP and the synthesis of poly(carboxybetaines) derived from P4VP and having the maximum degreeof quaternization of 95 %, were presented in previousstudies [11, 12]. In addition, by dialysis and lyophilization, the poly(carboxybetaines) were highly purified.The chemical structures of precursor and the two derivatives were presented in Figure 1, and

were confirmed by means of $^1$H-NMR spectroscopy. The spectral difference between the two derivatives of P4VP is that the units of 4VPB-1 generates a singlet signal at 5.2 ppm for $N^+$-$CH_2$-$COO^-$ protons, while the groups $N^+$-$(CH_2)_2$-$COO^-$ showed signals at 4.8 and 2.9 ppm [13].

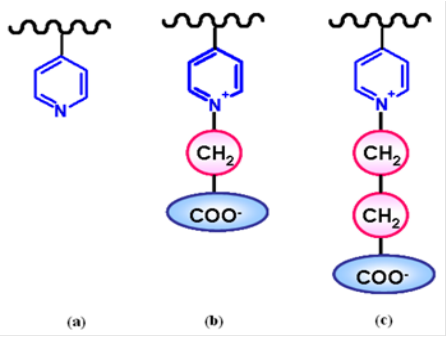



**Figure 1.**
Structural units of poly(4-vinylpyridine) (a), P4VPB-1(b) and P4VPB-2(c)

The solubility tests, based on the addition of 5 mL of solvent to a defined polymer quantity so that to obtain a diluted solution, stirring and forming of an homogeneous/inhomogeneous solution, showed that P4VPB-1 was insoluble in water, but soluble in salted water (with low molecular salt addition, e.g. NaCl). In contrast, P4VPB-2 was soluble in water with and without salt. Therefore, all micro-biological tests were performed on 0.5 M NaCl aqueous solutions.

The salted aqueous solutions of poly(carboxy-betaines) were prepared with purified water from a Millipore (Billerica, MA, USA) Simplicity UV apparatus, at room temperature, and then stirred about 24 h for homogenization. In all cases, the solvent and the polymer solutions were filtered by 0.02 μm, and 0.2 μm Whatman filters, respectively.

For antimicrobial susceptibility tests of the poly-(carboxybetaines), quality control organisms from the American Type Culture Collection were used. The strains were *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923). The growth medium for bacteria (Mueller-Hinton agar) was prepared in the laboratory according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [14].

The viability cell test with resazurin (BDH Ltd., U.K.) for *S. aureus* (ATCC 25923) strain allowed the determination of minimum inhibitory concentration (MIC) of P4VPB-2.

*Viscometry*

The viscometric measurements were performed using an Ubbelohde viscometer at 25.0 ± 0.05ºC (flow time of 168 s for distilled water). All viscometric measurements were achieved in triplicate and the average values were plotted. The type of viscometric behaviour for poly(carboxybetaines) in the presence of various concentrations of NaCl was established from reduced viscosity ($\eta_{red}$) plots.

*Kirby-Bauer cylinder-plate diffusion method*

Based on Kirby-Bauer diffusion method, the results of antimicrobial susceptibility of poly(carboxy-betaines) P4VPB-1 and P4VPB-2 were reported as diameters of inhibition zones (mm). The results of cylinder-plate method depend on the diffusion of antimicrobial agent from vertical steel cylinders placed on the surface of inoculated agar medium. For this test, the bacterial inoculum was obtained from 18 h cultures and pre-incubated at 37°C. Three millilitres of suspension with about $10^5$ CFU/mL were spread onto Petri plates containing Mueller-Hinton agar medium. After 10 min, the excess was removed by aspiration with a pipette, and then plates were left for 30 min to allow the micro-organism to adhere to the medium. Next, two sterile steel cylinders (internal diameter of 0.5 cm and height of 1 cm) were placed on each Petri plate. Then 0.2 mL of the test solution was pipetted into each cylinder. For each poly(carboxybetaine), the test solutions included about 50 mg/mL and 25 mg/mL, respectively. For antibacterial test, the polymers were dissolved in aqueous solutions of 0.5 M NaCl (a component of nutritive culture medium). To evaluate the bacterial growth, the plates were incubated for 24 h at 37°C, and next day, the diameter of the inhibition area around each cylinder was measured. Since the size of the inhibition zone depends on the concentration and the diffusion rate of the anti-microbial agent, the degree of sensibility and the growth rate of the microorganism, our results were compared to the control sample (solvent).

*Resazurin test*

The viability of bacteria after antimicrobial agent action was evaluated with resazurin (a redox indicator known as the main component of Alamar Blue). This simple, fast, sensitive and accurate test reveals the metabolic reducing potency of blue resazurin to pink resofurin by viable cells. Initially, for this purpose, 80 μL of culture medium (Muller-Hinton Broth, MHB) and 10 μL of 24 h bacterial culture with about $10^5$ CFU/mL, were placed in each well of a microplate. Then, 100 μL of tested polymer solution (P4VPB-2 in 0.5 M NaCl aqueous solution) with a concentration of 12 mg/mL, 6.4 mg/mL or 3.1 mg/mL were added. For control solution, the microplate wells contained only MHB and solvent (0.5 M NaCl aq. sol.). After 20 h incubation of microplate at 37ºC, that contained the test and control solutions in triplicate, 10 μL of resazurin (0.01% w/v solution prepared in sterile distilled water) were added to each well. After another 1 hour incubation in the presence of resazurin, the qualitative results were interpreted as positive for bacterial growth in the case of pink solutions (due to resofurin), but negative for bacterial growth in

the case of blue solutions (due to the unmetabolized resazurin).

## Results and Discussion

*Viscometric properties*

The poly(carboxybetaine) with a methylene group between the quaternary ammonium ($N^+$) and carboxylate ($COO^-$) groups was insoluble in water, but soluble in salty water. In contrast, the poly-(carboxybetaine) with two methylene groups between $N^+$ and $COO^-$ was soluble in water with and without salts. The dissolution in the presence of salts can be explained as follows: the zwitterionic polymers, andthus the poly(carboxybetaines), do not have counterions that neutralize the charge on the macromolecular chain as in the case of polyelectrolytes. The neutralization is possible between the positively charged quaternary ammonium group and carboxylategroup in the following ways: inter-chain associations, intra-chain associations between different structural units or within the same structural units. The last one determines the so-called internal salt structures ("inner salt").

In case of P4VPB-1, the neutralization occurs mainly by inter-chain associations that determine the formation of ionic three-dimensional network that finally leads to insolubility. The added salts penetrate the network and their ions neutralize the charge of zwitterionic group, causing the destruction of the three-dimensional network and inducing the polymer solubility. Also, in the case of P4VPB-2, the neutralization between $N^+$ and $COO^-$ is achieved predominantly within the same structural units, that makes poly(carboxybetaine) P4VPB-2 water soluble (Figure 2).

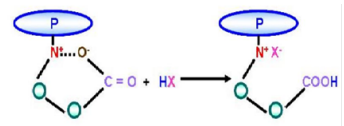



**Figure 2.**
Protonation of poly(carboxybetaine) P4VPB-2

The viscometric measurements for P4VPB-1 and P4VPB-2 solutions focused on the variation of reduced viscosity($\eta_{red}$) with the salt concentration ($c_s$) at a constant polymer concentration (1 g/dL) (Figure 3). It was observed that the values of reduced viscosity of poly(carboxybetaine) solutions in NaCl increase with the salt concentrations ($c_S$) and with the distance between the two charges $N^+$ and $COO^-$, respectively. In the literature, this behaviour is known as "anti-polyelectrolyte" effect [15].

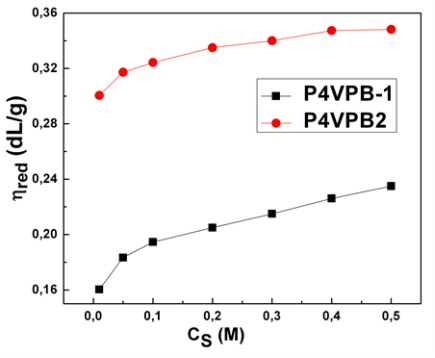



**Figure 3.**
Reduced viscosity of poly(carboxybetaines) at 25°C and various NaCl concentrations ($c_{polymer}$ = 1 g/dL)

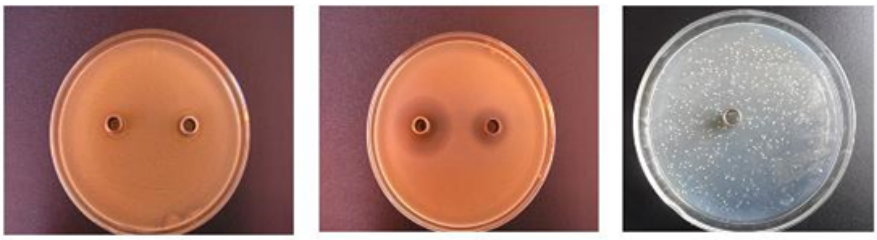
The previous studies have shown that the reduced viscosity did not vary with the concentration of polymer solution which indicated that the poly-(carboxybetaines) chains had rigid spheres conformations [16].

*Antibacterial susceptibility*

The polymers containing pyridinium ring or its derivatives are known as useful biomaterials due to their potential antibacterial activity [17, 18]. Regarding the interaction mechanism between the charged polymers (namely, polybetaines) and bacteria, it was demonstrated that after penetration of the bacterial wall, the rings of pyridinium or imidazolium could have formed polycomplexs with bacterial DNA [19, 20].

The antimicrobial potential of poly(carboxybetaines) was tested in aqueous salted solution (0.5M NaCl) because this medium is favourable for the extended conformation of macromolecular chains, and decreases the bacterial cell surface hydrophobicity. The growth medium, bacteria age and bacterial cell structure are the main factors that influenced the hydrophobicity of bacterial species [21]. Generally, based on different chemical composition of bacterial structural components, *S. aureus* is considered as a hydrophobic microorganism, but *E. coli* as a hydrophilic one. The presence of the salts (e.g. sodium chloride) will increase the exopolysaccharide production of bacteria, resulting in a faster decrease in the cell surface hydrophobicity if the exopolysaccharides were predominantly neutral or hydrophilic [22].

In the case of P4VPB-1 and P4VPB-2, the interactions between positive charges of pyridinium ring and negatively charged membranes of bacteria were predominantly electrostatic. In addition, in a little extent the hydrophobic interactions could appear between the hydrophobic regions of antimicrobial agents and the zwitterionicphospholipids (electrically neutral) from bacterial membrane surfaces.



(a) (b) (c)

**Figure 4.**

Diameters of inhibition zones of poly(carboxybetaines) against *Staphylococcus aureus* compared with control: (a) 0 mm for P4VPB-1 ($c_1$ = 50.6 mg/mL; $c_2$ = 25.2 mg/mL); (b) 27 mm and 15 mm for P4VPB-2 ($c_1$ = 51.7 mg/mL; $c_2$ = 26.2 mg/mL); (c) 0 mm for solvent (as control)

Generally, the polymers of pyridinium derivatives were highly effective agents against Gram-positive bacteria. In our study, P4VPB-1 did not inhibit the growth and the multiplication of *E. coli* or *S. aureus* most probably due to the shorter spacer between $N^+$ and $COO^-$ groups (Figure 4 and Table I).

On the other hand, the ethylene spacer from P4VPB-2 improved flexibility of macromolecular chains, and favoured the interactions with the bacterial cell components. All these ensured the appearance of the inhibition zones of 15 - 27 mm against *S. aureus* if the concentration of the antimicrobial agent was about 25 - 50 mg/mL.

**Table I**

Diameters of inhibition zones for P4VPB-1, P4VPB-2 and solvent (0.5 M NaCl)

| Sample | Solution concentration (mg/mL) | Diameter of inhibition zone (mm) | |
|---|---|---|---|
| | | ***E. coli* (G -)** | ***S. aureus* (G+)** |
| P4VPB-1 | 50.6 | 0 | 0 |
| | 25.2 | 0 | 0 |
| P4VPB-2 | 51.7 | 0 | 26.6 ± 1.14 |
| | 26.2 | 0 | 15.2 ± 0.84 |
| Solvent (as control) | - | 0 | 0 |

Plates were prepared using different concentrations of tested polymers. Zones of inhibition were measured on sets of 5 plates.

*MIC determination*

Over time, the resazurin test was used to assess the microbiological contamination of the foods and the medical devices, *in vitro* proliferation and the citotoxicity of different cellular types [21, 23-29]. In all these studies, the cell cultures recognize the resazurin from their environment as an external factor that must be metabolized. For this purpose, a series of cytoplasmic or mitochondrial enzymes from the cellular entity play the role of proton donors in the reduction reaction of resazurin to resofurin.

In our study, the resazurin test was used as "gold standard" to find the minimum inhibitory concentration of P4VPB-2. In this way, the results from cylinder plate method for *S. aureus* cell viability in the presence of P4VPB-2 were confirmed. Experimentally, three different concentrations of polymer were tested (12 mg/mL, 6.4 mg/mL and 3.1 mg/mL) and compared with solvent (0.5 M aqueous solution) as control. Also, the assay was realized in triplicate. Consequently, the results of the qualitative assessment of resazurin metabolism inbacterial cells were translated in a colour change of solutions from micro-wells, and allowed recording the MIC of 6.4 mg/mL for P4VPB-2 against *S. aureus*.

## Conclusions

The poly(carboxybetaines) P4VPB-1 and P4VPB-2 were soluble in aqueous solutions with low molecular salt addition (NaCl) and exhibited an "anti-polyelectrolyte effect". The spacer length and hydrophobicity of polymer chains influenced the antimicrobial potential. Thus, the poly(carboxy-betaine) P4VPB-1 did not prove antimicrobial action against any of tested microorganisms most probably due to a lower flexibility of pendant groups in bacterial culture medium. In case of P4VPB-2, a more extended conformation of macro-molecular chains has ensured the interactions with bacterial wall of tested Gram positive bacteria were efficient against it.

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