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Tuning anti-microbial activity of poly(4-vinyl 2-hydroxyethyl pyridinium) chloride by anion exchange reactions

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Abstract A series of new bioactive polymers with pendant choline analogous group was prepared by anion exchange reaction direct at the quaternary nitrogen of the polycation. Poly(4-vinyl 2-hydroxyethyl pyridinium) chloride was prepared in situ by simultaneous polymerization and quaternization of 4-vinyl pyridine with 2-chloroethanol that also acts as catalyst. The counter anion (Cl^-) of the polycation was exchanged by anion exchange reaction with Br^-, ^-OH, ^-SH, NO_3^-, BF_4^- or CF_3COO^-. Evidence of anion exchange was obtained by the characterization of the resultant polymers. The nature of the counter anion has profound effect on their properties including strong anion-dependent anti-microbial activity against bacteria and fungus. Polymer containing ^-OH was observed to be the most potent anti-microbial agent with the lowest minimum inhibitory concentration against both the classes of microbes studied.

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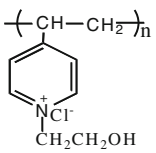
1 Introduction

Poly(4-vinyl pyridine) [poly(4-VP)] has reactive pendant pyridine moieties which can be modified via post reactions at the tertiary nitrogen. Quaternization with various alkylating agents enlarges the spectrum of its end-uses. The polypyridinium cation with variable structure of the alkylating/quaternizing agent possesses anti-microbial, catalytic, emulsifying and water-softening properties [1, 2]. Polypyridinium ions offer the advantage of high local concentration of the active groups, and are thus more effective than low molecular weight analogues. There are many reports on the properties of the quaternary ammonium, dialkylimidazolium ion or *N*-alkylpyridinium derivatives. These are technologically very attractive materials, and are reported as polymer supported reagents [3], CO_2 adsorbents [4, 5], and anion exchange membranes for fuel cells [6], polymer electrolytes [7, 8], biosensor [9], enzyme support [10], polymer surfactants [11, 12], and antimicrobial agents [13–18]. Anti-microbial activities of pyridinium polymers are affected by various aspects of polymer structure. These include molecular weight, nature of counter anions and length of the alkyl chain [19]. The antibacterial activity was found to be affected by the structure of the counter anion with the low activity for a counter anion, which tends to form a tight ion-pair high for those facilitating ionic dissociation to free ions. The anti-microbial properties were reported to follow the order: hloride > tetraflouride > perchlorate > hexafluorophosphate, which could be correlated with the solubility products of the polymers [20]. The peptide secondary structure, self-assembled nanostructures, and surface activity were observed to depend strongly on the type of anion [21].

Polymers having pyridinium as single or one of the components are good candidates as anti-microbial agents,

however, these are not good membranes agents and the preparation of vinylpyridinium monomer is not possible due to the spontaneous polymerization of 4-VP yielding polypyridinium cation in the polymer chain. The in situ spontaneous polymerization of 4-VP in the presence of an alkylating agent is an effective method to prepare fully quaternized polymer. The alkylating agent acts both as reactant and catalyst. The polymers formed by this procedure are fully quaternized [22]. The structure of polypyridinium cation can be changed by the use of different alkyl halides. The alkylation with haloalkanes has three main advantages. One, a wide range of haloalkanes is available at low cost. Two, the substitution reaction on the haloalkanes occurs smoothly at a reasonably low temperature. Three, the halides anions can easily be exchanged by metathesis reactions to generate a variety of polymers. In view of the above, in the resent study we prepared a range of poly(4-VP) based active polymers by its quaternization with 2-chloroethanol to generate a bioactive polymer with the pendant choline analogous group, as shown:



Choline has $[(CH_3)_3N^+(CH_2CH_2OH)^-OH]$ structure. It is an important constituent of the fat metabolism, and acetyl choline is involved in nerve impulse. In the present article, apart from the synthesis and characterization of active polymers having choline analogous pendant group, we report their anti-microbial activity against fungi *Aspergillus niger* and *Mucor circenelliods*, and bacteria *Bacillus coagulans* (*BTS-3*). To modify the anti-microbial activity of poly(4-vinyl 2-hydroxyethyl pyridinium) chloride, the counter anion (Cl^-) was replaced with Br^-, OH^-, SH^-, NO_3^-, BF_4^- and CF_3COO^-. Thus, a series of polymers was obtained by carrying out simple anion exchange reaction direct on the polycation [23]. Polymers thus prepared were characterized by nitrogen analysis, differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR), nuclear magnetic resonance (NMR), and X-rays diffraction (XRD) to get evidence of anion exchange. To the best of our knowledge, such a series of polypyridinium based polymers has not been reported earlier. Pyridinium polymers are non-toxic toward mammalian cells, hence safe for use in biomedical applications. These are useful in biomedical applications including coating of catheter, etc., and also as gels for skin infections and burns.

2 Experimental

2.1 Materials

4-Vinyl pyridine (Merck, Schuchardt, Germany), 2-chloroethanol, NaBr, NaOH, NaSH, $NaNO_3$, $NaBF_4$ and CF_3COOK (S.D. Fine, Mumbai), were used as received.

2.2 Synthesis and quaternization of poly(4-VP) with 2-chloroethanol

Polymerization and quaternization of poly(4-VP) was carried out by treating it with 2-chloroethanol in 1:5 weight ratio to ensure the maximum extent of the reaction. The two components were taken in glass ampoules, which were sealed and placed at 50°C for 36 h. There after, the product was extracted with 1,4-dioxane to remove the unreacted 2-chloroethanol. The polymer was dried in a vacuum desiccator. The polycation poly(4-VP-*N*-CH_2CH_2OH) was abbreviated as $[PVPy]^+$ and the polymer as $[PVPy]^+Cl^-$.

2.3 Anion exchange reactions

$[PVPy]^+Cl^-$ was dissolved in a minimum amount of the double distilled water to make a saturated solution. The saturated solutions of NaBr, NaOH, NaSH, $NaNO_3$, $NaBF_4$, or CF_3COOK were prepared separately in the double distilled water. The solution of the 'as prepared' $[PVPy]^+Cl^-$ was added to each of the salt solution, and after initial gentle stirring these were allowed to stand for 30 min. The precipitates were collected and dried in vacuum desiccator. The supernatant from each set was used to analyze Cl^-. Thus, a series of seven polymers was obtained, and these polymers were designated as: $[PVPy]^+Br^-$, $[PVPy]^{+-}OH$, $[PVPy]^{+-}SH$, $[PVPy]^+CF_3COO^-$, $[PVPy]^+BF_4^-$, and $[PVPy]^+NO_3^-$.

2.4 Characterization of polymers

All the polymers were characterized by DSC (851 Mettler Toledo), FTIR spectroscopy (Nicollet 5700 FTIR Spectroscope), NMR spectroscopy (Bruker DPX 300), XRD (Bruker D8 Advance), and nitrogen analysis (Carlo Erba 1150 analyzer).

2.5 Antimicrobial activity of polymers

The polymers were characterized by studying their antimicrobial activity against fungus (*Aspergillus niger* and *Mucor circenelliods*) and bacteria (*Bacillus coagulans BTS-3*). The cultures of the fungi were maintained on

potato dextrose agar slants which contained extract (boiled potato 20%, dextrose 1%, and agar 2%) at 3.5 pH, while that of bacteria was maintained on the nutrient agar slants. The media used for fungi was potato dextrose broth, and for bacteria was nutrient broth. 2.5 ml of media was taken in test tubes and autoclaved at 121°C and 15 psi. A known amount of different polymers were added to the test tubes those were inoculated with 2 × 105 pores. There after these were incubated at 37°C for 72 h. The minimum amount of the polymers that inhibits the growth of bacteria/fungi was calculated by the minimum inhibitory (MIC) method [24]. All tests were performed in duplicate, and control experiments without polymers were set.

2.6 Results and discussion

Spontaneous polymerization of 4-VP in the presence of the 2-chloroethanol is a simple method that results in full quaternization to yield $[PVPy]^+Cl^-$. Thus, no ambiguity remains regarding the structure of the quaternized product [22]. The resultant polymers prepared by the simple tuning of anion exchange show anion-dependent properties, as a simple polymer analogous reaction becomes basis to generate a range of useful properties on the same polymer. The polymers prepared from $[PVPy^+]Cl^-$ were characterized by various techniques to get evidence of anion exchange and the effect of the nature of anion on the polymer properties.

2.7 Evidence of anion exchange by characterization of polymers

Results of nitrogen and sulfur analysis support the anion exchange (Table 1). %N found in all the polymers confirms that the exchange of Cl^- with different anions occurred with high rate of conversion. The supernatant obtained from the anion exchange reactions exhibited presence of Cl^-. Taking %N (6.97) found in $[PVPy]^+Cl^-$ as benchmark, %N was found to decrease more prominently in the case of heavy anions such as Br^-, BF_4^- and CF_3COO^-, while more %N was found in the polymer after exchange. BF_4^- and NO_3^- form crosslinks with the polymer chains; hence the result was lower Cl^- extraction in these cases as compared to the other anions. The representative DSC curves of some polymers are presented in Fig. 1. The poly(4-VP) has high glass transition (T_g) at 182°C (by heating) and at 140°C (by cooling) [25]. In the present case, an anion effect was observed on the thermal behavior as T_g varied in a narrow range from 10.2°C of $[PVPy]^+{}^-OH$ to 34.3°C of $[PVPy]^+CF_3COO^-$. T_c of poly(4-VP) decreased on quaternization and it was lower at 118.53 and 129.28°C. $[PVPy]^+{}^-OH$ and $[PVPy]^+{}^-SH$ have closely related structure, and out of all the samples studied, the area under the crystallization peak in the DSC curve is very high for these two with the largest area for the former and closely followed by the latter. The thermal behavior of the other two closely related polymers $[PVPy]^+Cl^-$ and $[PVPy]^+Br^-$ also shows similarities. From the area under the crystallization peak, the heat of crystallization is the highest for $[PVPy]^+{}^-OH$ and the lowest for $[PVPy]^+BF_4^-$. It is thus apparent that the polymer structure in these polymers is anion-dependent as on anion exchange the arrangement and the intermolecular

Table 1 %N found in polymers

Polymer	% of nitrogen	
	Calculated[a]	Found
[Poly(4-VP)-CH_2CH_2OH]$^+$ Cl^-	7.57	6.97
[Poly(4-VP)-CH_2CH_2OH]$^+$ Br^-	6.086	6.01
[Poly(4-VP)-CH_2CH_2OH]$^+$ BF_4^-	5.91	5.31
[Poly(4-VP)-CH_2CH_2OH]$^+$ ^-OH	8.38	8.17
[Poly(4-VP)-CH_2CH_2OH]$^+CF_3COO^-$	5.3	5.16
[Poly(4-VP)-CH_2CH_2OH]$^+NO_3^-$	13.21	12.29
[Poly(4-VP)-CH_2CH_2OH]$^+$ ^-SH	7.65	7.16

[a] Based on the theoretical value for the repeat unit $[PVPy]^+X^-$ for 100% conversion, where X^- is the counter anion other than Cl^-

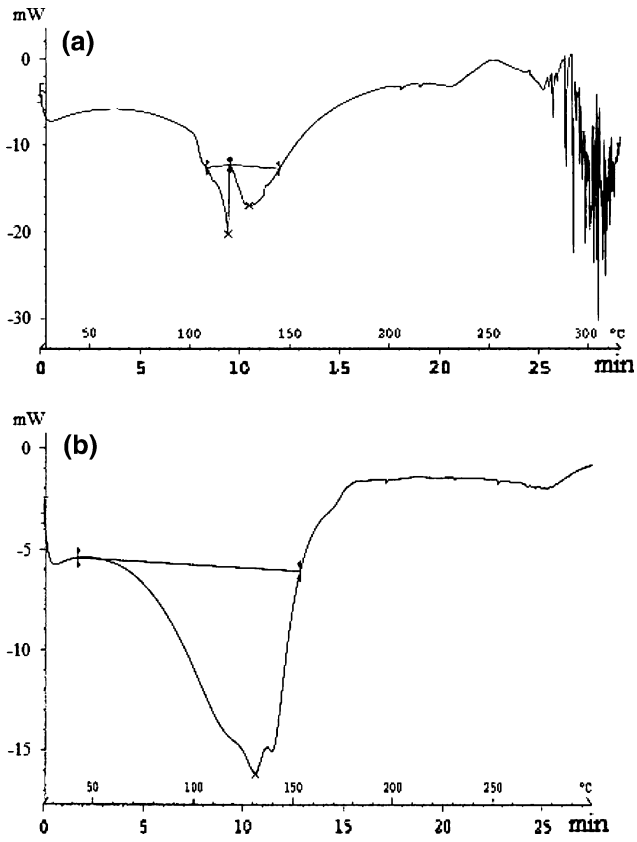


Fig. 1 DSC curves of (**a**) $[PVPy]^+Cl^-$, and (**b**) $[PVPy]^+OH^-$

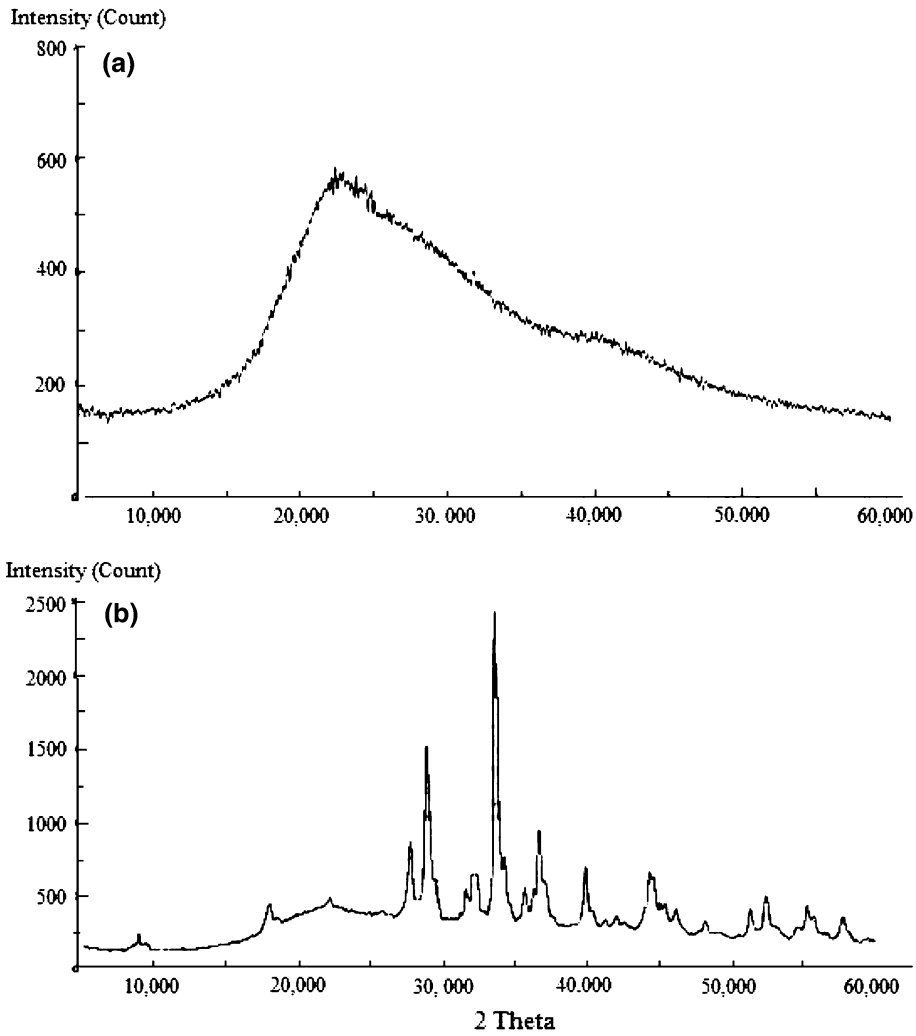


Fig. 2 XRD patterns of (**a**) $[PVPy]^{+}Cl^{-}$, and (**b**) $[PVPy]^{+}OH^{-}$

association between the polymer chains changed in a significant manner.

Two representative XRD spectra are presented in Fig. 2. The pure poly(4-VP) has three prominent peaks—the small one at 5.2 degree (2θ), the most intense at 10.9 degree (2θ) and intensity of the third peak is in between the two other peaks at 20.6 degree (2θ) with the respective d-spacing (nm) at 1.7, 0.81, 0.43 [25]. In the present study, the 5.2 degree (2θ) peak is absent in the XRD of all the polymers, but the peak at 10.9 degree (2θ) appears as small, but sharp peak in the $[PVPy]^{+-}OH$ and $[PVPy]^{+}NO_3^{-}$. XRD of $[PVPy]^{+}Cl^{-}$ has a broad peak around 20 degrees (2θ) (Fig. 2a). This peak has high intensity in the XRD of $[PVPy]^{+}BF_4^{-}$, but it significantly lower in the XRD of the other polymers, especially in the case of $[PVPy]^{+-}OH$ where it gets obliterated (Fig. 2b). Thus, on quaternization, and later by the anion exchange, the basic peaks of poly (4-VP) are weakened or are totally obliterated indicating change of the crystalline or generally change of the amorphous contents. Activation of SiO_2–CIO–Na_2O glass composition by the cation-exchange process results in a better control of the material treatment without affecting bioactivity or biocompatibility [26]. $[PVPy]^{+-}OH$ has crystalline phase and the JCPDS search match showed that the crystalline phase is monoclinic trona. The weak crystalline phase observed in $PVPy^{+}BF_4^{-}$ did not match with any JCPDS file. The FTIR spectra of some polymers are presented as Fig. 3. In the spectrum of $[PVPy]^{+}Cl^{-}$, the characteristic peak of the pyridinium cation prominently appears near 1,645 cm^{-1}, 1,200–1,080 cm^{-1} due to $–CH_2–CH_2–$ group, and the sharp bands at 669 cm^{-1} indicating the presence of Cl^{-} (Fig. 3a). On exchange of Cl^{-} with different anions, the spectra differ only below 2,000 cm^{-1} because in this region each anion gives its characteristics peak. The F–B stretching at 1,065 cm^{-1}, C–F stretching at 1,190 cm^{-1}, O–NO_2 stretching at 1,660 cm^{-1}, and a band at 1,384 cm^{-1} is attributed to the NO_3^{-} in the vicinity of the aromatic ring. The high intensity peak at 1,100 cm^{-1} along with a peak at 580–540 cm^{-1} region confirms the presence of BF_4^{-}. The FTIR spectrum of $[PVPy]^{+-}OH$ has

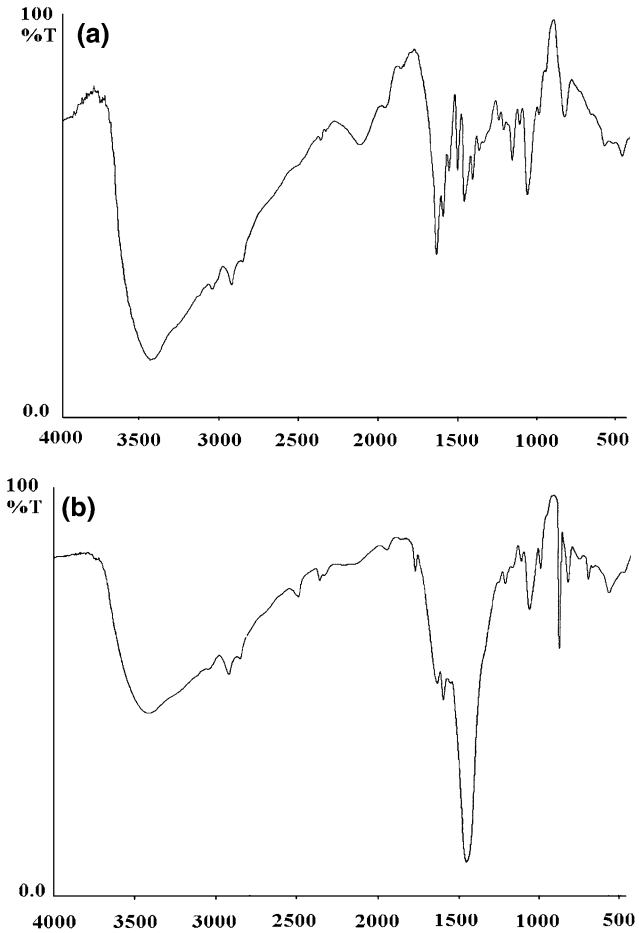


Fig. 3 FTIR spectra of (**a**) $[PVPy]^+Cl^-$, and (**b**) $[PVPy]^+OH^-$

broad bands at 1,200–1,070 cm^{-1} due to the presence of ^-OH in the polymer (Fig. 3b).

^{1}H-NMR spectra of the candidate polymers were recorded in DMSO-d6. The broad and intense signals in the region of 2–4.0 ppm are due to the protons on $>N^+$-CH_2CH_2OH, overlap with the $–CH_2–CH–$ protons of the polymeric chain. The signals at 6.4 and 8.2 ppm are assigned to the ring protons. On quaternization, the peaks of aromatic protons shifted to the higher δ value, i.e., at 8.4 and 6.7 ppm because of the dishelming effect produced by the quaternary pyridinium atom of the ring. Signals at 6.3–6.7 ppm and 6.7–8.0 ppm are due to Roth and meta protons with respect to pyridinium N, respectively. The spectrum of $[PVPy]^+Cl^-$ shows hyperfine structure of very fine and sharp lines (not presented). Since the XRD pattern of this sample has indicated amorphous phase, the hyperfine structure in NMR spectra is ascribed to low interactions among the molecules in the liquid phase compared to the strong interactions of the molecules in the $[PVPy]^{+-}OH$ or $PVPy^+BF_4^-$. The ^{1}H NMR spectra of $[PVPy]^{+-}OH$ shows both sharp and broad signals in the aromatic region of 6–8.50 ppm. The signals are more intense in the regions of 6.7–8.5 pm compared to 6.7–6.3 ppm. The ^{1}H NMR broad pattern is an indicator of the strong interaction and ordered alignment even in the liquid state of this polymer. The ordered structure of this polymer is also confirmed by XRD. The ^{1}H NMR spectra of polymer containing BF_4^- and NO_3^- indicate broad signals in the region of 6.6–8.5 ppm similar to the amorphous structure with a small degree of crystalline, as also observed in their XRD. This is again indicative of the strong interaction with no degree of orderliness in these polymers.

2.8 Anti-microbial activity of polymers

Pyridinium polymers are non-toxic to the mammalian cells; hence these are useful in biomedical applications. The cationic part of the polymer, anions, or the combination of polymer with a specific anion has been reported to exhibit cytotoxicity [27]. The anti-microbial activity of these polymers is due to the cationic part. It has been established in the preceding section that anion effect is specific and strong enough to alter the properties of the precursor polymer, and its effect on the anti-bacterial properties is collateral. We studied the anti-microbial activity of the synthesized polymers under the physiological conditions, against three representative microbes, *Aspergillus niger* and *Mucor circenelliods* (fungi) and *Bacillus coagulans BTS-3*. The fungi are known to cause serious pulmonary infections, while some Bacillus species cause bacteremia/septicemia, endocarditis, meningitis, and infections of wounds, ears, eyes, respiratory tract, urinary tract, and gastrointestinal tract. The aim of the present study is to use these polymers in vivo as graft on the biomedical devices such as catheters, and in vitro as gels against the skin infections. Hence, an investigation into their anti-microbial activity against both bacteria and fungi was desirable to ascertain their activity spectrum.

The antimicrobial activity was calculated by the minimum inhibitory concentration (MIC) method, i.e., the lowest concentration of the compound that inhibited the visible growth of microbes. The MIC (the least concentration of an anti-microbial agent to prevent microbial growth) values of different polymers against the above-cited microbes are presented in Fig. 4. Against *Mucor circenelliods*, the weakest anti-microbial action or the highest MIC of 8.33 mg/ml was observed for the $[PVPy]^+Br^-$ as against 4.17 for the precursor polymer, while $[PVPy]^{+-}OH$ exhibited the highest activity [Fig. 4a]**.** In the case of *Aspergillus niger*, $[PVPy]^{+-}OH$ and $[PVPy]^{+-}SH$ exhibited comparatively lower activity than observed for the other fungus [Fig. 4b]. As compared to others, the polymers containing halogens anions are the least effective against these fungi, hence tuning of anti-microbial property by anion exchange was useful exercise.

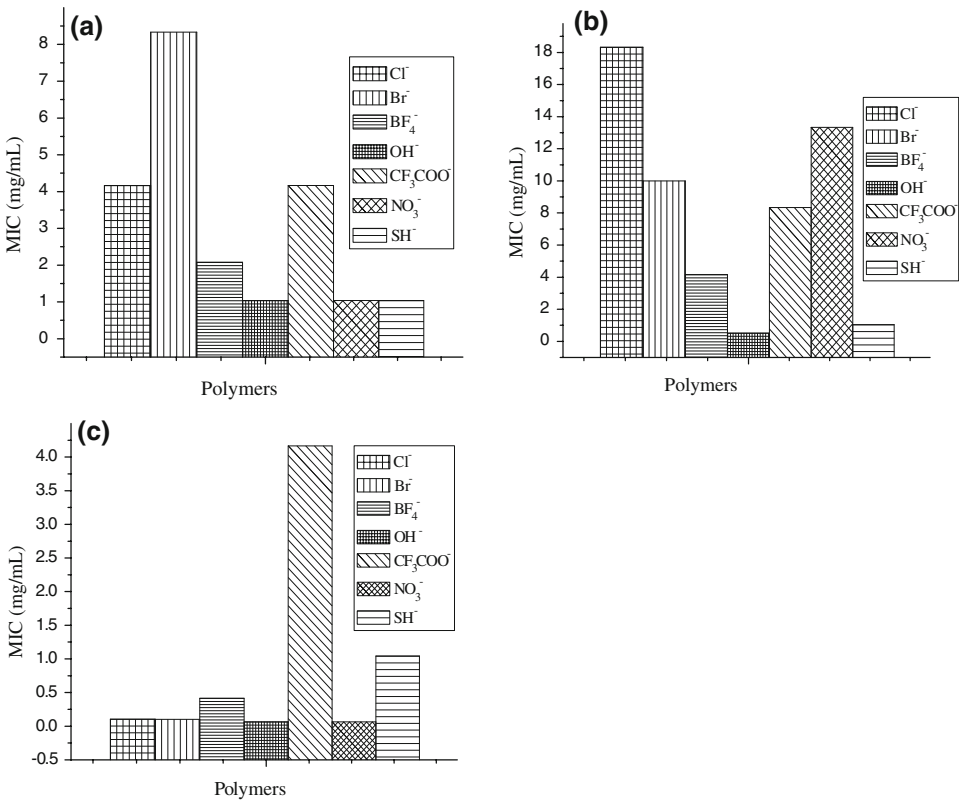


Fig. 4 MIC of polymers against (**a**) *Mucor circenelliods*, (**b**) *Aspergillus nigar*, and (**c**) *Bacillus coagulans BTS-3*

Most of the polymers exhibited far stronger anti-bacterial activity than anti-fungal activity. A strong anti-bacterial activity with the lowest MIC of 0.065 mg/ml was exhibited by most of these polymers, but for the one having CF_3COO^- counter ion. The strongest action or the lowest MIC of 1.04 mg/ml was obtained for the $[PVPy]^{+-}OH$, $[PVPy]^{+-}SH$ and $[PVPy]^{+}NO_3^{-}$ [Fig. 4c]. Out of the seven polymers studied, $[PVPy]^{+-}OH$ exhibited the strongest anti-microbial activity against fungi as well as bacteria than other polymers, thus structure–property relationship is established from the observed results. These polymers show stronger action against *Bacillus coagulans* than the fungi studied. At the physiological pH, cell walls of all the microbes become negatively charged. Bacterial cell wall contains negatively charged phosphatidylethanolamine (70%) as the major component; hence activity of the cationic polymers is more against the bacteria than fungi.

The mechanism of anti-microbial activity is driven first by the electrostatic forces between the cationic groups of polymer and negatively charged cell membrane, and thereafter the van der Waals interactions between the hydrophobic moieties of both the polymer chains and phospholipids follow [28]. When microbe come in contact with the polymer surface and attempt to grow, the peptidoglycan cell wall synthesis is immediately interrupted. This results in the disruption of cell membrane and microbe biofilm formation is prevented. The interactions at the polymer surface exemplify carpet mechanism [29], as the alkylated pyridinium polymers provide smooth surface for interaction with microbes [20, 30]. Effective close contact with the anti-bacterial surface has also been demonstrated to reduce bacterial adhesion or proliferation onto the material surface or even close to the material itself [26, 31]. The mechanism in such cases is by the release of the active metal ions/part which results in the formation of pits on the surface of the material, whereas in the present case, the mechanism is similar to the carpet mechanism that resembles membrane disruption by detergents. The differences in the MIC values are explained by the anion effect on the structure of the polymers as discussed in the preceding section under DSC and XRD studies. Since the changes in the polymer structure after anion exchange are anion dependent, the extent of interactions between the cationic centre of the polymer and the cell walls of the microbes are also anion dependent. Hence, $[PVPy]^{+-}OH$ is the most potent polymer against the microorganisms studied. The presence of the pendant choline analogous moiety at the cationic toxicophore is active in fat metabolism and it also contributes to the anti-microbial activity by the polysoap action [2]. The other reason that is attributed to the high efficacy of $[PVPy]^{+-}OH$ in the present study is its high solubility in water that results in its effective interaction with the microbes [20]. On the other hand, polymers such as $[PVPy]^{+}BF_4^{-}$ do not easily dissolve in water due to the crosslink formation, hence high MIC values and low anti-microbial action.

3 Conclusions

Poly(4-vinylpyridinium-2-hydroxyethyl) chloride was prepared in stiu by simultaneous polymerization and quaternization using 2-chloroethanol as catalyst and reagent. A range of the active polymers was prepared by the simple anion exchange reaction at the quaternary nitrogen of the polycation and the extent of anion displacement was high. The anion effect on the properties of different polymers was inferred from the various analytical tools. There is strong evidence to suggest that structure of an anion has profound affect on the structure–property profile of the polymers, especially changing the polymer morphology. Thus, an easy tuning of the polymer properties was achieved by simple anion exchange that results in potent broad spectrum anti-microbial properties of these polymers. The structure–property relationship is evident as the polymer having $^{-}$OH as counter ion exhibited the strongest anti-fungal and anti-bacterial activity with MIC values of 1.04 and 0.52 mg/ml against the fungi *Mucor circenelliods* and *Aspergillus niger,* respectively, and 0.065 mg/ml against the *Bacillus coagulans*.

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