Antibacterial Activity of Novel Insoluble Bead-Shaped Polymer-Supported Multiquaternary Ammonium Salts

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ABSTRACT: This study describes the effect of antibacterial activity of newly reported five different novel insoluble bead-shaped polymer-supported multiquaternary ammonium salts (PM quats) viz., bis-quat, tris-quat (2 Nos.), tetrakis-quat, hexakis-quat containing two, three, four, and six quaternary ammonium groups, respectively. The presence of number of quaternary ammonium groups in each salt was established already through Fourier-transform infrared spectroscopy, thermogravimetric analysis, scanning electron microscopy, and chloride ion analyzes. The antibacterial activities of these five different PM quats against three different bacteria viz., *Staphylococcus aureus, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were investigated by serial dilution and spread plate method and compared the same with a monoquat containing single quaternary ammonium group. The extent of antibacterial activity has been measured in terms of colony forming units (CFU) at different time intervals. The observed results show that all the PM quats exhibited excellent-antibacterial activity against each bacterium. On the basis of the CFU values, the antibacterial activity was found to increase from bis-quat to hexakis-quat, which reveals that the activity of PM quats increases with increase in the number of quaternary ammonium groups. The mechanism of interaction of quats with bacterial cytoplasmic membrane has been explained as an adsorption-like phenomenon. The reusability of highly active hexakis-quat against *Staphylococcus aureus* was studied and the activity was found to reduce after first cycle. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 117: 3673–3678, 2010

Key words: quaternary ammonium salts; antibacterial activity; *Staphylococcus aureus*; *klebsiella pneumoniae*; *pseudomonas aeruginosa*

INTRODUCTION

Quaternary ammonium salts (quats) containing ammonium (N^+) as central onium ions have found wide-spread applications in several areas viz., industrial, environmental, and biological chemistry. Quaternary onium salts are commonly used as phase-transfer catalysts, antimicrobial agents, surfactants, detergents, wetting agents, emulsifying agents, skin antiseptics, and disinfectants.[1–3] Generally soluble form of quats are used to exert excellent antimicrobial activity in comparison with other antimicrobial agents because of their effective cell membrane penetration properties, low-toxicity, good environmental stability, nonirritation, low-corrosivity, extended residence time, and biological activity.[4,5] In 1940, Kuhn and Bielig suggested that quaternary ammonium cations might act on the bacterial membrane.[6] The end result of the interaction between bacteria and a quat is the leakage of intracellular components because of disruption of the membrane functionality.[7,8] There are several soluble quats with strong-antibacterial activity reported in literature, but they are highly toxic to the environment as protection from them was short-lived because of the difficulty in controlling the rate of diffusion into the bacteria, besides recontamination problems.

As a remedial measure, during the last two decades, continuous effort has been made to develop antimicrobial polymers in which the quats are anchored to a polymeric backbone via covalent bond. These polymer-supported quats (polyquats) were able to kill bacteria that were resistant to other types of cationic antibacterials.[9–11] They could exert antimicrobial activities in a controlled manner by mere contact in water without releasing the reactive agents. They can be used without contamination of the substrate; they can be removed easily and used repeatedly. The first study for the antibacterial action of polyquats was reported in 1928 by Hartmann and Kagi.[12] The surface of polymer-supported beads matrix grafted with polyquats is found to contain more positively charged cations and hence, they exert a strong-adhesive force toward negatively charged bacteria, and thus physically inhibit the subsequent growth of bacteria.[13] These

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polymeric antimicrobial agents are nonvolatile, chemically stable, and do not permeate through the skin. Several polymers such as polystyrene,[14] chitosan derivatives,[15] and poly(glycidyl methacrylate)[16] containing quats have been synthesized, and their antimicrobial properties have been reported.

Numerous studies have proved that the insoluble polyquats possess high-antibacterial efficiency against different bacteria.[17–21] Popa et al.[22,23] reported the synthesis of quaternary ammonium and phosphonium salts grafted on cross-linked polymer-supports and their application as antibacterial agents for removal of bacteria from water. Although several types of soluble and insoluble antibacterial monoquats were reported, they suffer from lower activity and irritant side effects because of the availability of a single quaternary ammonium group. To exclude this problem, soluble multiquats containing more than one quaternary ammonium group were developed. The synthesis and antibacterial activity of various bis-quats have already been reported by Devinsky et al.,[24,25] Kourai et al.,[26] and Ohkura et al.[27,28] in which they proved that bis-quats can exhibit higher antibacterial activity than monoquats. Similar studies, viz., the antibacterial activity of bis-quats like N,N,N′,N′-tetraalkyl-N,N′-bis(alkylbenzyl)-N,N′-2-butynylene-1,4-bis(ammonium halide),[29] decamethylene-bis-4-amino quinaldinium halide,[30] and bis-(2-dimethylaminoethyl) glutarate[31] were also reported recently. Though these soluble multiquats have proved to be effective to provide more antibacterial activity, but still their employability is very less because of recontamination problem. Insoluble multiquats containing multiquaternary ammonium groups can overcome this problem, but the detailed literature study reveals that there are no reports available on these insoluble multiquats. Hence, in this study, it is attempted to examine the effect of antibacterial activity using five different bead-shaped polymer-supported multiquaternary ammonium salts (PM quats) containing two, three (2 Nos.), four, and six quaternary ammonium groups and compared with monoquat viz., polymer-supported benzyltriethylammonium chloride (PSBTEAC).

EXPERIMENTAL

Chemicals

Gelatin (Lancaster, Morecambe, Lancashire, England) boric acid (SRL, Mumbai, India), polyvinyl alcohol (Lancaster), styrene (Lancaster), vinylbenzylchloride (Lancaster), divinylbenzene (Lancaster), AIBN (Lancaster), triethylamine (SRL), 3,5-dimethylphenol, sulfuryl chloride, thionyl chloride, triethylmethane tricarboxylate (Lancaster), lithium aluminum hydride (Merck, Mumbai, India), phosphorus trichloride (Lancaster), sodium hydride (Lancaster), para-hydroxy acetophenone (Lancaster), formaldehyde (37–41 %), diethanolamine (Merck), calcium oxide, 1-bromopropane, magnesium sulfate, sodium hydroxide (SRL), potassium carbonate (Merck), tetrahydrofuran, dimethyl formamide (SRL), dimethylsulphoxide (SRL), dichloromethane (SRL), acetonitrile (SRL), methanol (SRL), and acetone (SRL), diethyl ether (SRL) were used as provided.

Preparation of PM quats

The procedures for the preparation of five different heterogeneous PM quats viz., bis-quat of polymer-supported 3,5-bis(triethylammoniummethylene chloride) benzene (PSBTEACB),[32] tris-quat of polymer-supported 4-(2,2,2-tris[triethylammonium methylene chloride]-1-hydroxyethyl)phenylether (PSTTEACHPE),[33] tris-quat of polymer-supported 1,3,4-tris(triethylammoniummethylene chloride)-2-methylbenzene (PSTTEACMB),[34] tetrakis-quat of polymer-supported 3,5-bis(bis[triethyl ammoniummethylene chloride]aminomethyl) benzene (PSBBTEACAB),[32] and hexakis-quat of polymer-supported 3,5-bis(4-(2,2,2-tris[triethylammoniummethylene chloride]-1-hydroxy ethyl) phenyl ether (PSBTTE ACHPE)[32] salts containing two, three (2 nos), four, and six quaternary ammonium groups, respectively, were already reported in the literature. By adopting these procedures, we prepared all the five different PM quats (Fig. 1). The structure and presence of quaternary ammonium groups in each PM quat has again established with Fourier-Transform Infrared Spectroscopy, Thermogravimetric analysis, Scanning electron microscopy (SEM), and [chloride ion] analyzes.

SEM analysis

Preparation of sample

The surface morphology of newly synthesized five different PM quats such as PSBTEACB, PSTTEACHPE, PSTTEACMB, PSBBTEACAB, PSBTTEACHPE, and the monoquat PSBTEAC were analyzed individually through SEM using representative mesh size of +120 – 140. The nonconductive polymeric materials are made into conductive by giving uniform platinum coating. The respective PM quats were spread on the surface of double sided adhesive tape, one side of which was already adhered to surface of a circular-copper disc pivoted by a rod. JEOL JSM-6360 auto fine-coating ion sputter was used for the platinum coating under identical experimental conditions.

Antibacterial test

The culture of bacterial pathogens viz., *Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* was obtained from Fermentation laboratory,

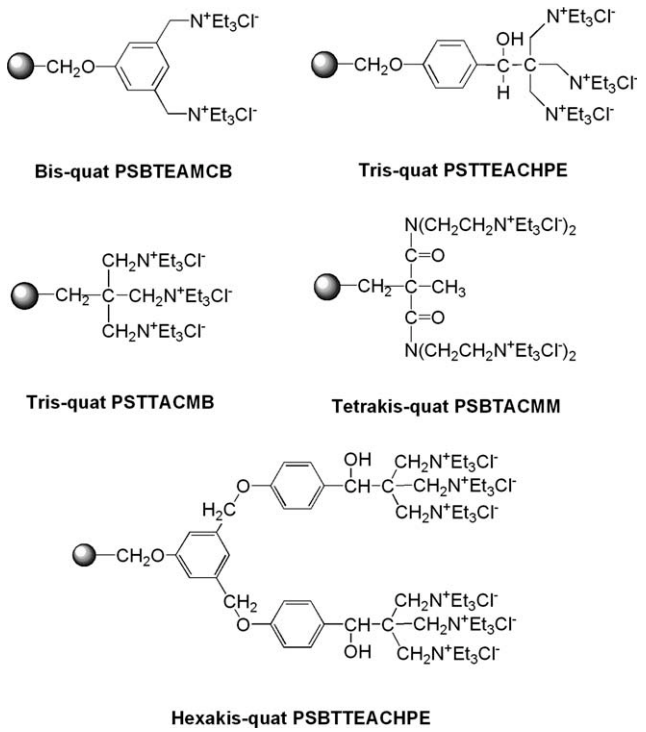

Figure 1 Structure of PM quats.

CAS Botany, University of Madras. One loopful of mother culture from each bacterium was transferred into 20 mL of nutrient broth containing peptone (0.1 g), NaCl (0.1 g), Beef extract (0.02 g), and water (20 mL) and incubated for 24 h at room temperature under shaking condition. After the incubation, the spore load was calculated using hemocytometer. The inoculum was diluted to get 1×10^6 cells/mL.

To examine the antibacterial activity of all the five types of PM quats, they have been employed individually under identical experimental conditions against three different bacteria. That is, in a 250 mL Erlenmeyer flask, 0.2 mmol of each polymer-supported quat and sterile water were added individually under aseptic condition and the respective mixture was stirred at room temperature for 1 h. Then 1 mL of each test bacteria viz., *Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* was added individually to each PM quat solution at room temperature, and then stirring was continued. At 1 h intervals, 1 mL of respective PM quat/bacteria samples were collected, and the number of colony forming units (CFU) was determined by the standard decimal serial dilutions method.[35] From each diluted solution, 1 mL was seeded to a petri plate and then 10 mL of nutrient gelose (melted and cooled at 45°C) was added. The mixture was stirred to get homogenized solution and then left to solidify at room temperature. The obtained plates were incubated for 24 h at 37°C and the number of colonies/mL was determined. After each use, the polymer-supported quat was washed with sterilized water, separated by filtration, and then sterilized at 100°C for 24 h. The stability of the polymer-supported quats was tested using the repeated treatment described to the study of antibacterial activity.

Statistical analysis

All the data were statistically evaluated with SPSS/10.0 software. Hypothesis testing methods included one-way analysis of variance followed by least significant difference test $p < 0.05$ was considered to indicate statistical significance. All the results were expressed as mean ± S.D.

RESULTS AND DISCUSSION

Surface morphology study of PM quats using SEM

SEM analysis was carried out using the five different PM quats having same mesh size of –120 + 140. The observed results on the surface morphology were compared with that of monoquat PSBTEAC. The magnified SEM images of single-bead for all the quats were already reported.[32–34] The comparative microgram results reveal that the surface of PSBTEAC show a smooth homogeneous surface without any tiny nodules. Whereas, in the case of PM quats, tiny nodules were noticed on the surface of the beads and thus exhibits a heterogeneous surface. The availability of tiny nodules is found to be less in bis-quat PSBTEACB and little more in tris-quat PSTTEACMB followed by tris-quat PSTTEACHPE and tetrakis-quat PSBBTEACAB. Finally the higher order of heterogeneity or more number of nodules was noticed for hexakis-quat PSBTTEACHPE. This type of observation confirmed the availability of more number of quaternary ammonium groups in the respective PM quat.

Comparative antibacterial activity of PM quats

The *in vitro* antibacterial activity of all the five different PM quats and the monoquat PSBTEAC were studied individually in bath system by adopting the serial dilution and spread plate method against three different bacteria including *Staphylococcus aureus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa.* The extent of antibacterial activity has been estimated through their ability to kill bacteria in water and measured in terms of CFU that is, a measure of viable bacterial cells in a sample per mL in which a colony represents an aggregate of cells derived from a single-progenitor cell. The CFU value was measured at different time intervals of exposure time (i.e.) from 1 to 6 h using all the six PM quats irrespective of bacteria. The observed results show that

all the PM quats exhibited antibacterial activity against each bacterium. However, based on the CFU values, it is observed that the order of antibacterial efficiency of the PM quats is monoquat PSBTEAC < bis-quat PSBTEACB < tris-quat PSTTEACMB < tris-quat PSTTEACHPE < tetrakis-quat PSBBTEACAB < hexakis-quat PSBTTEACHPE. This observation clearly reveals that the antibacterial efficiency of PM quats increases as the number of quaternary ammonium groups increases. The increase in the number of quaternary ammonium groups should increase the density of positive charge (N^+) of quats, which in turn promotes the aggregation of active group on the bacterial surface, thus provide stronger-driving force to reach the cytoplasmic membrane of bacterial cells.[17] That is, the positively charged quaternary ammonium groups have attracted the negatively charged bacterial cell surface via electrostatic interaction and thus inhibits the growth of bacterial cells.

The interaction of quats with cytoplasmic membrane can be established by drawing a plot using log (CFU) as a function of the exposure time against the bacteria viz., *Staphylococcus aureus* (Fig. 2), *Klebsiella pneumoniae* (Fig. 3), and *Pseudomonas aeruginosa* (Fig. 4). On increasing the exposure time, the concentration of active, viable bacterial cells is found to be decreased in each plot thus proving the antibacterial action. The observed plots irrespective of bacteria and PM quats also indicate that the antibacterial action depend on the number of quaternary ammonium groups in each PM quat and their function of the exposure time. That is, the activity of each PM quat irrespective of bacteria was found to increase steadily. More specifically, the polymer-supported hexakis-quat PSBTTEACHPE was found to be more active than the other PM quats irrespective of bacteria, and this observation directly confirmed the availability of more number of quaternary ammonium groups in hexakis-quat PSBTTEACHPE. Further, it is also understood from the values that the activity of the PM quats was distinctly higher for *Staphylococcus aureus* than for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Fig. 5).

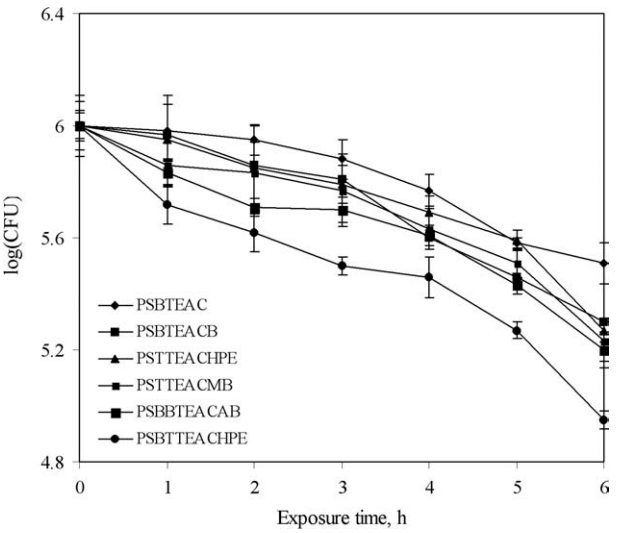

Figure 3 Plots of log(CFU) versus exposure time using PM quats against *Klebsiella pneumoniae*.

Mechanism of antibacterial activity

The mechanism of action of polyquats involves penetration or adsorption of the salts into the viable cells and disruption of the integrity of the cell membrane, like a needle bursting a balloon.[36] The antimicrobial activity of polyquats may be because of the

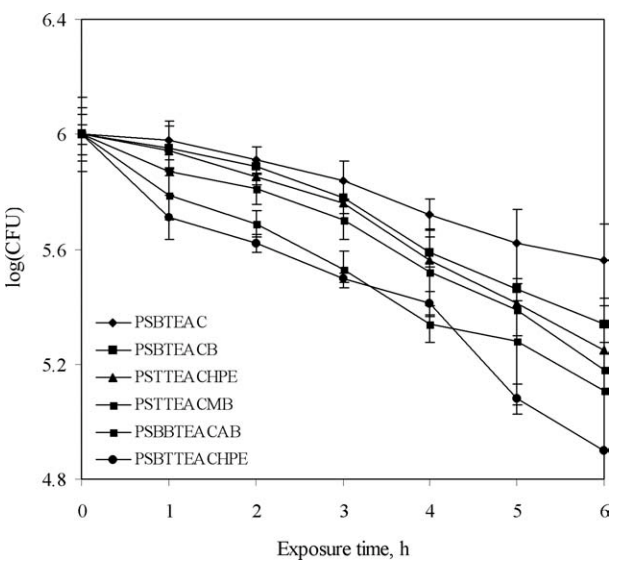

Figure 2 Plots of log(CFU) versus exposure time using PM quats against *Staphylococcus aureus*.

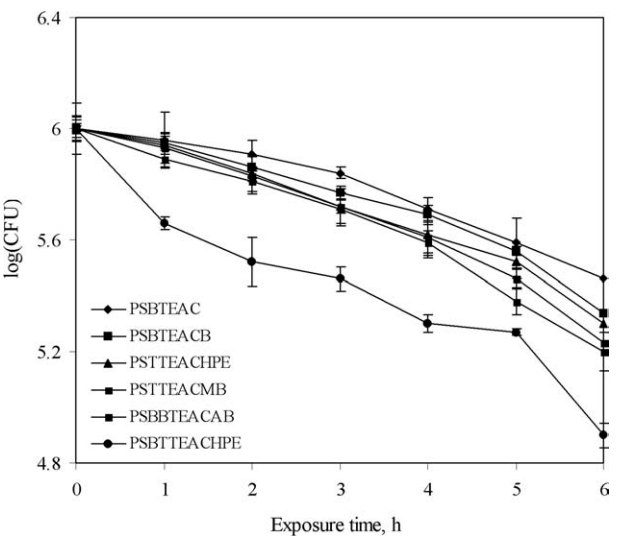

Figure 4 Plots of log(CFU) versus exposure time using PM quats against *Pseudomonas aeruginosa*.

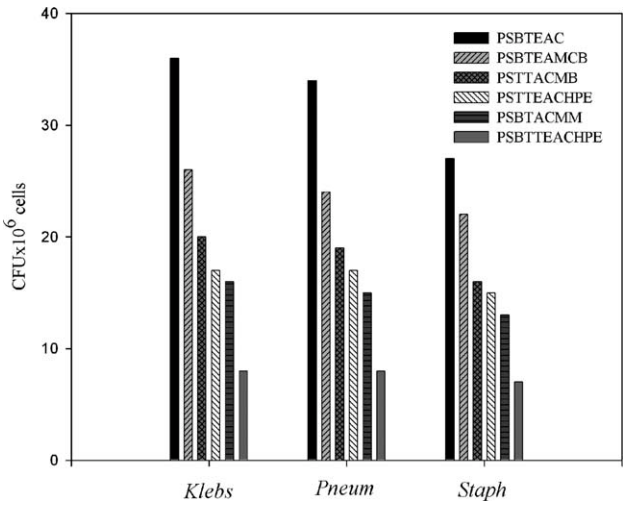

Figure 5 Comparative antibacterial activity of PM quats after 6 h of exposure.

ion exchange between the positive charges on the polymeric surface and structurally critical mobile cations within the membrane.[37] They kill the bacteria by damaging the cytoplasmic membrane and inhibiting membrane associated functions such as nutrient transport and energy transduction. The highly charged groups concentrated on a polymer backbone lead to a high-electrostatic potential along the chain.

After absorption, part of the polymer backbone diffused through the cell wall and the quaternary groups interact with the cytoplasmic membrane and causes the death of bacteria. The penetration is intensely affected by the interaction of ammonium group with the cytoplasmic membrane and mobility of the functional group inside the cell membrane. Akihiko et al.[17] reported that the positive charge density (N^+) is an important key point to the antimicrobial ability of polymeric quaternary salts and suggested that high-positive charge density may enhance the driving force which in turn presents high-antibacterial activity. In this study, the newly synthesized salts contain more number of quaternary ammonium groups and they should provide stronger-driving force to reach the cytoplasmic membrane by the interaction of more number of quaternary ammonium groups with the membrane causing cell death. Based on the above discussion, we propose the following sequence of elementary processes, which are key factors for activity analysis of PM quats.

i. Adsorption of positively charged polymer-supported quaternary ammonium compounds onto the negatively charged bacterial cell surface.
ii. Diffusion or penetration of polyquats through the cell wall of bacteria.
iii. Binding to the cytoplasmic membrane (lipid or protein) followed by its disruption.
iv. Release of cytoplasmic constituents that is, intracellular low-molecular-weight material (K^+ ions).
v. Degradation of proteins and nucleic acids (DNA and RNA).
vi. Cell wall lysis caused by autolytic enzymes and death of the cell.

Reuse of hexakis-quat PSBTTEACHPE

To study the stability of PM quats, the reusability of highly active hexakis-quat PSBTTEACHPE against *Staphylococcus aureus* was conducted for repeated cycles. That is, the compound used in the first cycle was regained via filtration and sterilization process, and then it was reused for second cycle by maintaining the same experimental conditions. Likewise, this hexakis-quat PSBTTEACHPE was repeatedly studied against the same bacteria till the fifth cycle. The CFU results obtained in different time intervals for each cycle were converted into their percentage of reduction. The plot has been derived using percentage of reduction of bacterial cells as a function of exposure time. From the plot (Fig. 6), it is understood that the activity of hexakis-quat PSBTTEACHPE had significantly decreased that is, on comparing with the first cycle, $\approx$ 35% of activity was reduced in the second cycle and in the subsequent third, fourth, and fifth cycle the activity was not reduced sharply rather activity remains approximately constant as observed in second cycle. This is because, in the second cycle, sizeable amount of quaternary ammonium groups may get dislodged owing to the disturbance occurred in the porosity of the poly(styrene) based

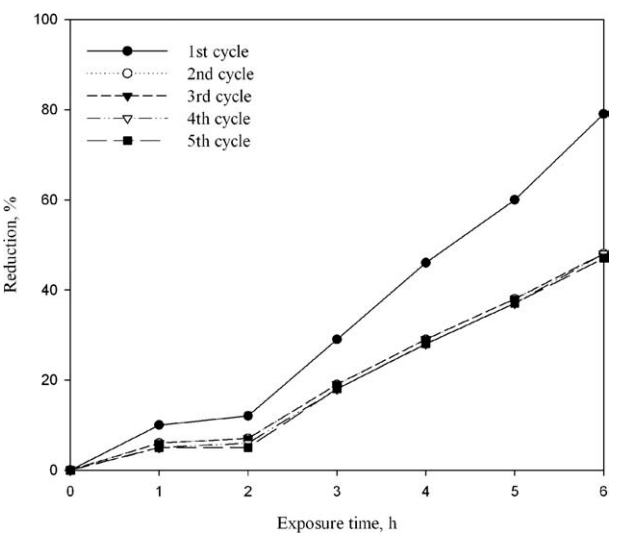

Figure 6 Plot of the reduction of surviving cells versus exposure time in repeated cycles using PSBTTEACHPE against *Staphylococcus aureus*.

polymer-supported matrices network. Particularly, after the first cycle, the PSBTTEACHPE quat was filtered and sterilized using water; thereby the polymer-supported network was poorly swelled or shrinked and thus diminishing the exposure of the active centers and reduced the antibacterial activity in the second cycle. Then, in the subsequent cycles (3, 4, and 5), the activity remained essentially unmodified, because of the change of more stable and compact porous texture of the poly(styrene) based polymer-supported matrix. Hence, the antibacterial activity remains almost similar as that observed in the second cycle.

CONCLUSION

We have prepared five different new bead-shaped PM quats containing two, three (2 nos.), four, and six quaternary ammonium groups, respectively. The *in vitro* antibacterial activity of all the five different PM quats and monoquat PSBTEAC against three different bacteria including *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* studied individually by adopting the serial dilution and spread plate method. The order of activity in terms of CFU values was observed as PSBTEAC $<$ PSBTEACB $<$ PSTTEACMB $<$ PSTTEACHPE $<$ PSBBTEACAB $<$ PSBTTEACHPE. This result shows that antibacterial activity of PM quats was dependent on the number of quaternary ammonium groups that is, the positive charge density. The study on reusability of hexakis-quat proves that there is loss in antibacterial activity ($\approx$ 35 %) after first cycle and the activity remains unmodified in the subsequent cycles. Hypothesis testing methods by one-way analysis of variance show that the results were statistically significant at $p < 0.05$.

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