Investigation of Solid Supported Dendrimers for Water Disinfection

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ABSTRACT: Polystyrene copolymer beads supported dendrimer was synthesized and investigated for its biological applications. Macroporous cross-linked polystyrene copolymer beads were synthesized using suspension polymerization. Two successive generations of di(chloroethyl) amine-type end group functionality was formed on the polystyrene copolymer beads. Elemental analysis, Fourier transform infrared spectra, and solid state ^{13}C NMR spectra were employed to characterize the polymer bound dendrimer. The polymer bound dendrimer was tested for antibacterial action against both Gram-positive and Gram-negative bacteria. The activity against both types of organism increased with an increase in the nitrogen atoms in the polymer back bone. The dendritic structure containing both amino and di(chloroethyl) groups showed significant reduction in the bacterial count when kept in 20 mL autoclaved water with bacterial cultures having an initial count in the range of 12–83 $\times$ 10^6 CFU/mL. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 1384–1391, 2012

Key words: macroporous; polystyrene; dendrimers; antibacterial; bacterial cultures

INTRODUCTION

Microbial contamination remains a challenging task for areas like health care, drugs, water purification, packaging, and storage sectors. Researchers are still in the search of antimicrobial materials with broad range of microbial activity. To overcome these hurdles, insoluble polymers containing antimicrobial functional groups are studied for their antimicrobial action.

Dendrimers are a new group of macromolecules synthesized in an iterative sequence of reaction steps in which further iteration leads to a higher generation molecule. The structural perfection of dendrimers in comparison with other polymers makes them very attractive and novel materials in many areas of technology development.[1] The dendrites are hyper branched three-dimensional molecules having host-guest entrapment properties. Dendrimers are unique, well-defined structures emanating from a central core region with outer branches for holding very high number of functional groups. The use of dendrimers for the study and modulation of biological processes is gaining popularity.[2] Dendrimers are attractive due to their ability to display multiple copies of surface groups which interest researchers to study them for antimicrobial applications. Generally, dendrimers displaying biocidal properties are terminated with antimicrobial agents like quaternary ammonium,[3] carbohydrates,[4] and peptides.[5–7] In recent times, dendrimers have already attracted increasing attention for their applications in many fields including combinatorial chemistry,[8] electrochemistry,[9] sensors,[10] photochemistry,[11] nanoparticle synthesis,[12] water purification,[13,14] antimicrobial,[15] dye decolorization,[16] catalyst,[17,18] drug delivery systems, and gene transfection[19] and in biomedical fields.[20] Owing to their structure and availability of many end groups, dendrimers have attracted researchers for their antibacterial applications.[3] The study of halogen and amine containing dendritic architecture for their biological application is new for investigation, as the halogen bound dendrimers have been hardly studied for their antibacterial efficacy. The exact mechanism of antimicrobial action of such large molecules is unclear as they have lower permeation rate through the bacterial cell membranes. As the biocidal action requires an interaction with the bacterial cell membrane, the activity might be due to the adhesion/adsorption of cells on the polymer surfaces. Such bacterial adhesions on the synthetic surfaces were reported earlier.[21] So functional groups and their antimicrobial activity yet remains an unsolved protocol. Biocidal action

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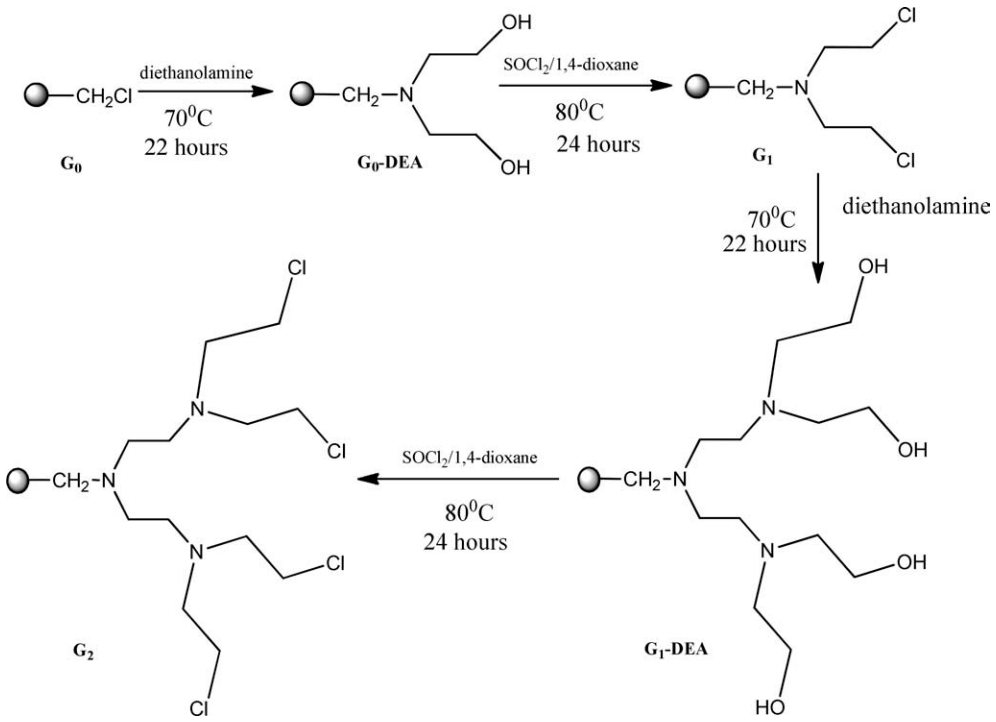

Scheme 1 Synthesis of dendrimer type resin.

requires interaction with the bacterial cell membrane which can be attributed as the permeability of bacterial cell wall, disruption of bacterial cell wall, electrostatic interactions,[22] and other van der waals force of attraction which are influenced by the functional groups of the dendrimer.

This study focuses on the evaluation of halogen and amine containing dendritic architecture for antibacterial application. The antibacterial activity was investigated against both Gram-positive and Gram-negative model organisms. The localization of varied active groups as well as that of the different number of functionalities from a single core has made them promising antibacterial agents. The Gram-positive cell wall contains several layers of peptidoglycan which are interconnected by side chains and cross bridges, which in turn, render rigidity to the bacterial cell. While in the Gram-negative bacteria, the cell wall contains very low amount of peptidoglycan in the periplasmic space and they are covalently linked to lipoproteins in the outer membrane. Owing to the low amount of peptidoglycan, the cell wall of Gram-negative bacteria can be easily disintegrated. This basic difference in the nature of the cell boundaries of the Gram-positive and Gram-negative bacterial cells poses a great challenge for establishing a common antibacterial agent. In this study, two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and two Gram-negative bacteria (*Escherichia coli and Pseudomonas aeruginosa*) have been investigated for ascertaining the antibacterial behavior of the dendrimer.

EXPERIMENTAL

Materials

Styrene and divinyl benzene were obtained from ς-Aldrich (USA). Diethanolamine, thionyl chloride, and heptane were purchased from FC Finar (India). 1, 4-Dioxane, aluminum chloride were purchased from S.D. Fine chemicals (India). Nutrient broth, bacteriological agar, and nutrient agar were purchased from Hi-Media Laboratories (Mumbai, India). The bacterial cultures of *S. aureus* ATCC 25923 (NCIM 5021), *B. subtilis* ATCC 6051 (NCIM 2920), *E. coli* ATCC 8739 (NCIM 2065), and *P. aeruginosa* ATCC 9027 (NCIM 2200) were procured from NCIM (Pune, India).

Synthesis of copolymer beads

63% w/w styrene and 7% w/w divinyl benzene were mixed with 30% w/w of a porosogenic agent. Benzoyl peroxide (1.0% of w/w of monomer mixture) was added to the reaction mixture and dispersed in the suspension medium. The copolymerization was carried out at 80°C for 3 h. The copolymer beads thus obtained were washed with hot water and dried at 60°C. The copolymer beads were later extracted with suitable solvent to remove the porosogenic agent. Further, chloromethylation of the copolymer beads was carried out as reported.[23]

Synthesis of dendrimer type resin

The dendritic architecture on copolymer beads was synthesized by following the reported method.[24] The

chloromethylated polystyrene copolymer beads was named G_1. Chloromethylated polystyrene beads were reacted with diethanolamine and the product obtained was diethanolamine groups supported on polystyrene beads (G_0-DEA). Elemental analysis showed 4.62% nitrogen. 6 g of G_0-DEA was swelled in 100 mL of 1, 4-dioxane for 30 min. To this, 24 mL of thionyl chloride was added drop wise for 24 min and further heated at 90°C for 24 h to get di(chloroethyl) amine type G_1. The resultant G_1 copolymer was transferred to the soxhlet's extraction apparatus for reflux extraction using heptane as a solvent and dried at 60°C. Five grams of G_1 copolymer was again reacted with 20 mL of diethanolamine at 70°C for 22 h to get diethanolamine functionalized G_1-DEA. Elemental analysis of G_1-DEA showed 5.34% nitrogen. Four grams of G_1-DEA was again further reacted with thionyl chloride to obtain G_2. G_2 copolymer was extracted in soxhlet apparatus using heptane as solvent and dried at 60°C. The functional conversion of the beads was determined using carbon, hydrogen, nitrogen (CHN) analysis. The synthesis of dendrimer type resin in given in scheme 1.

Characterization

The functional group modification on the copolymer beads was investigated with Perkin–Elmer FT-IR spectrometer (Model-FT-1730). CHN analysis was carried out using Perkin–Elmer-2400 CHNS analyzer to confirm the extent of functionalization after each modification. ^{13}C solid-state NMR spectra were acquired on Bruker Avance II 500 MHz spectrometer equipped with 4 mm CPMAS probe and samples were made to spin at 15 kHz of MAS frequency. ^{13}C spectra were acquired with cross polarization technique.

Antibacterial activity of dendrimer

The antibacterial activity of the halide terminated dendritic architectures G_0, G_1, and G_2 was tested against two Gram-positive bacteria *S. aureus* ATCC 25923 (NCIM 5021) and *B. subtilis* ATCC 6051 (NCIM 2920) and two Gram-negative bacteria *E. coli* ATCC 8739 (NCIM 2065) and *P. aeruginosa* ATCC 9027 (NCIM 2200). All the bacterial strains were routinely subcultured and maintained in nutrient broth at 37°C and were stored at 4°C in nutrient agar slants as stock cultures. All the cultures used for this study were grown aerobically in nutrient broth at 37°C for 24 h.

Plate method

Nutrient agar media was poured into each of the presterilized Petri dishes and was allowed to solidify. One hundred microliters of bacterial cultures were spread on separate plates uniformly. G_1 and G_2 copolymer beads were gently placed over the solidified agar gel with the help of a sterilized spatula. Further, all the plates were then subjected to incubation at 37°C for 24 h.

Test tube test

All the test tubes were presterilized before usage to avoid contamination. One hundred microliters of bacterial culture was added to test tubes containing 20 mL of autoclaved water. All the test tubes were labeled to indicate the culture it contained. The quantities of G_0, G_1, and G_2 copolymer beads were varied as 100 mg, 200 mg, and 300 mg to each of these test tubes containing *E. coli, P. aeruginosa, B. subtilis*, and *S. aureus* bacterial suspensions, respectively. The purpose of varying the quantities of each of the copolymer beads was to optimize the bacterial growth reduction with an increase in the copolymer weight. All the tubes were placed on a rotatory shaker platform at a speed of 120 rpm. One hundred microliters of supernatant fractions were withdrawn from each of these tubes and plated after every 30 min upto 4 h. The supernatant fractions were diluted 10 times for determining the bacterial reduction as a function of contact time.

Testing bacterial adhesion/adsorption on the solid supported dendrimer

Adsorption/adhesion of the bacterial cells over the copolymer can reduce the viable bacterial count compared with the actual killing, so the active copolymers beads were subjected to bacterial adhesion/adsorption testing. The G_2 dendrimer type copolymer beads equilibrated in the bacterial suspension were washed with saline and kept on the nutrient agar gel plate to check the bacterial adhesion/adsorption onto the copolymer beads.

RESULT AND DISCUSSION

The Fourier transform infrared (FTIR) and CHN analysis performed shows the generation of functional group having dendritic architecture over the copolymer bead. The ^{13}C solid-state NMR spectra also validate the changes after every functionalization. The amino ethyl halide type end group dendritic architecture was synthesized for studying its antibacterial activity.

FTIR spectral studies

The FTIR spectral analysis was performed to obtain the characteristic change in functional group after every reaction step given in Figure 1. The G_0 shows

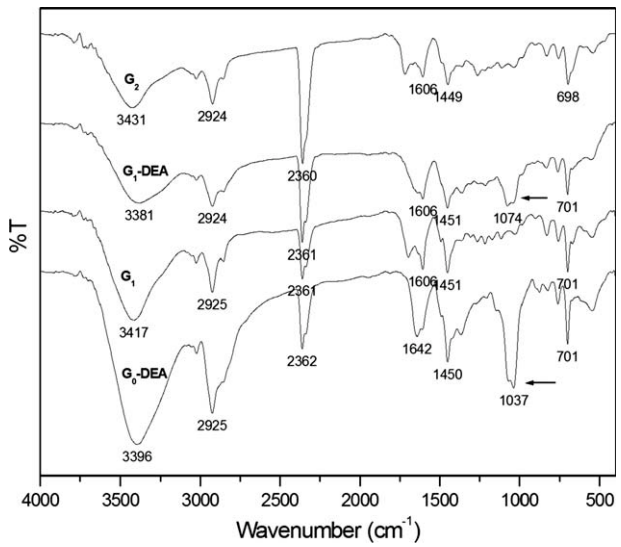

Figure 1 FTIR spectra of G_0, G_0-DEA, G_1, G_1-DEA, and G_2.

a characteristic peak at 675 cm^{-1} which disappeared in G_0-DEA and a new broad peak appeared at 1037 cm^{-1}, which is a characteristic peak of C—O bond indicating DEA introduction into the copolymer beads. This broad characteristic peak of C—O disappeared after G_0-DEA reacted with thionyl chloride. The same C—O characteristic broad peak reappeared at 1074 cm^{-1} after making G_1 react with DEA again. When G_1-DEA is again made to react with thionyl chloride, the C—O characteristic peak disappeared showing the conversion of functional group to C—Cl.

Solid state ^{13}C NMR spectral studies

Figure 2 shows the ^{13}C solid-state NMR spectra of styrene-divinyl benzene (SD), G_0, G_1, and G_2 from top to bottom. Peak corresponding to CH_2Cl site appears at around 55 ppm and its para position in the styrene ring is identified by a peak around 140 ppm marked by *e*. Substitution in CH_2Cl by di(chloroethyl)amine is expected to reduce the intensity of 55 ppm peak with introduction of a new peak around 60 ppm which can be clearly observed in G_1. Chloroethyl attached to N is expected to produce a peak with a chemical shift value of *f*, <55 ppm, marked with *g*. Other peaks in the spectra of G_1 is expected to shift by up to 2 ppm, hence broadening the overall spectrum and merger of *e* site with that of *c* and *d*. With further di(chloroethyl)amination increase in the intensity at 50 ppm peak can be observed.

Antibacterial tests

E. coli ATCC 8739 (NCIM 2065) had been selected as a fecal contamination indicator, *P. aeruginosa* ATCC 9027 (NCIM 2200) as a soil contamination indicator, *B. subtilis* ATCC 6051 (NCIM 2920), and *S. aureus* ATCC 25923 (NCIM 5021) as human influence indicators of water. The bacterial cultures were grown aerobically in nutrient broth at 37°C for 24 h and maintained as stock cultures on Nutrient agar slants at 4°C.

Bacterial susceptibility using plate method

To examine the susceptibility of all the test organisms, Nutrient agar plates were prepared and allowed to solidify. One hundred microliter samples of each of the bacterial suspensions cultured in nutrient broth were plated onto these plates uniformly. The plates were then supplemented with dendrimer, and then incubated further at 37°C, 24 h for the development of zone of inhibition. Figure 3 shows the zone of inhibition developed for the tested polymers.

Bacterial susceptibility using tube method

To determine the antibacterial effect of the newly synthesized dendrimer, they were equilibrated with

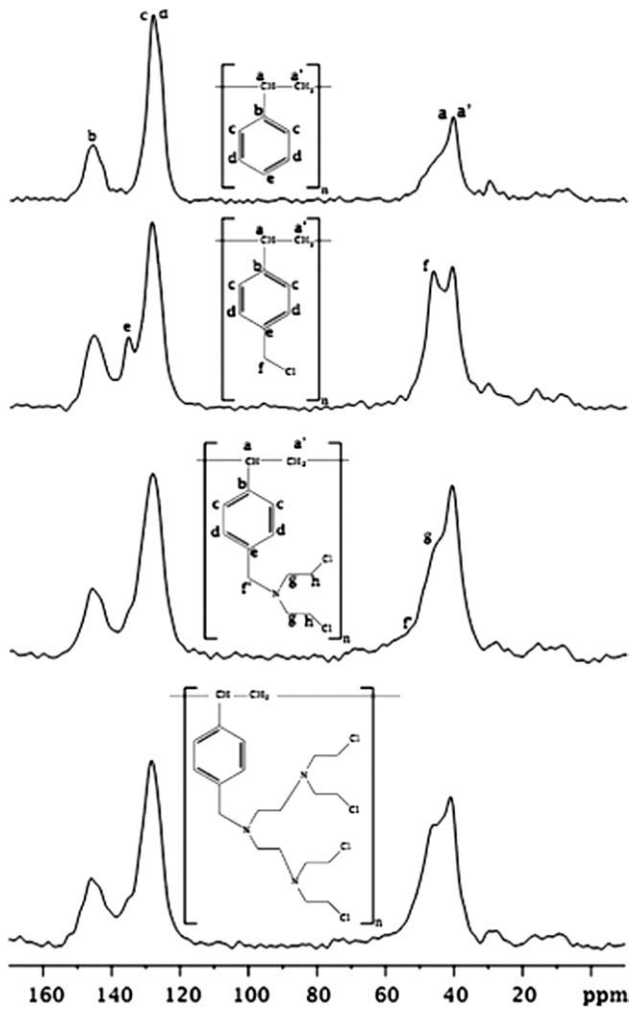

Figure 2 ^{13}C solid-state NMR spectra of SD, G_0, G_1, and G_2.

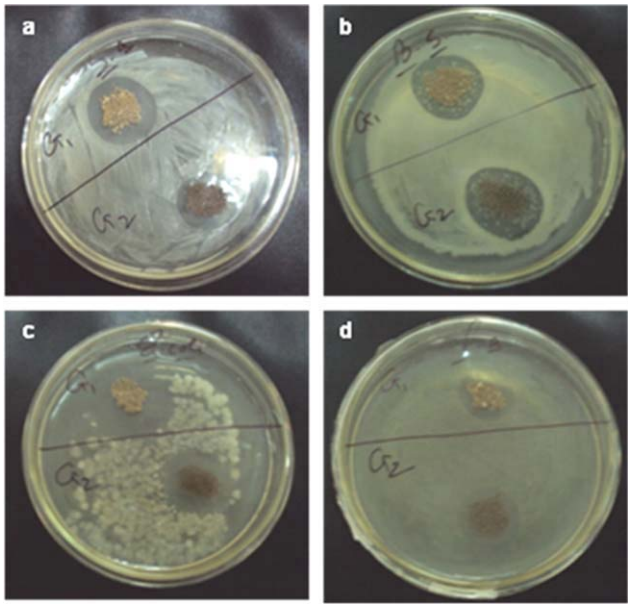

Figure 3 Plates showing zone of inhibition for G_1 and G_2 dendritic copolymer beads. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

20 mL of autoclaved water containing 100 μL bacterial suspension in each test tube. 100 mg, 200 mg, and 300 mg of each G_0, G_1, and G_2 dendrimers were added to each of the test tubes containing the bacterial suspensions of the four test organisms. The initial count of bacterial cultures varied from 14 to 84 $\times$ 10^6 CFU/mL. All the tubes were then kept on the orbital shaker platform with an aim to study the growth inhibitory effect of the dendrimers. Fixed aliquots of 100 μL were withdrawn from each tube after an interval of 30 min up to 4 h and plated on the nutrient agar plates to investigate the influence of contact time in killing the bacterial cells. The supernatant fractions were further subjected to a series of dilution for obtaining the bacterial count. The plates were then incubated and the colonies formed were counted.

G_0, G_1, and G_2 having alkyl chloride and amine functional group were investigated to study the influence of higher functionalization on the antibacterial activity. It was observed that G_0 exhibited lesser antibacterial effect as compared with G_1. This property can be attributed to the observation that with the introduction of (dichloroethyl) amine group on the copolymer bead, the antibacterial activity of

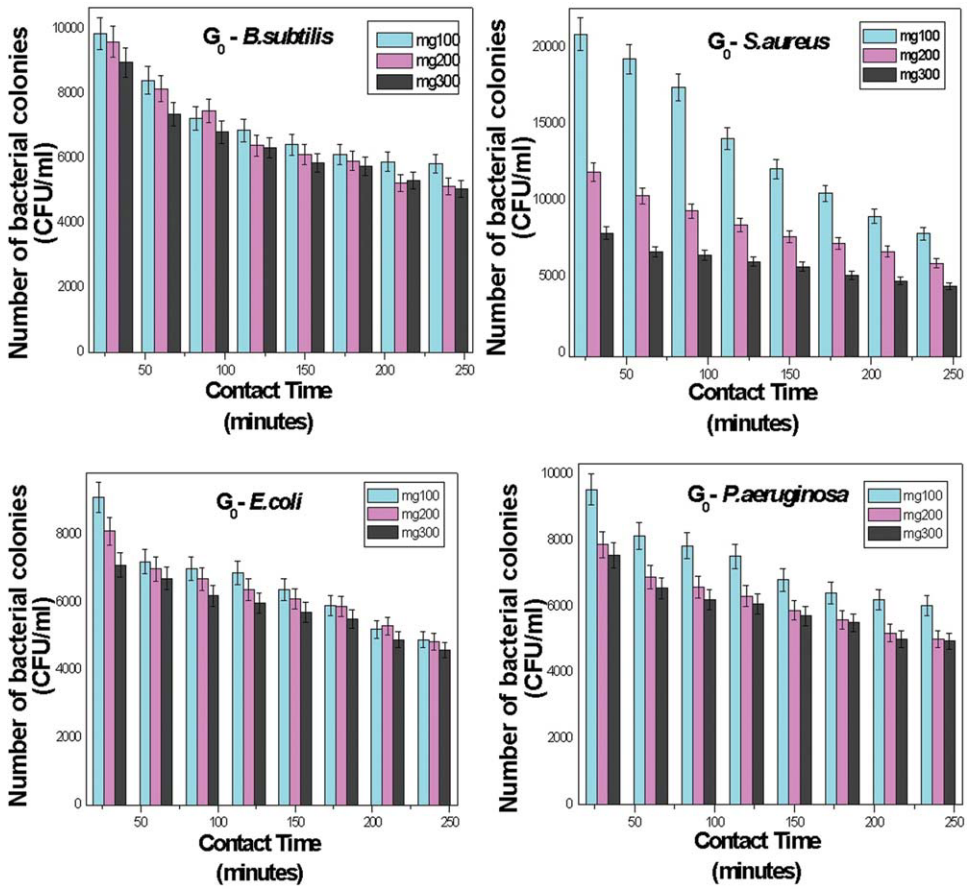

Figure 4 Graph showing the biocidal activity of G_0 copolymer bead against the four organisms. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

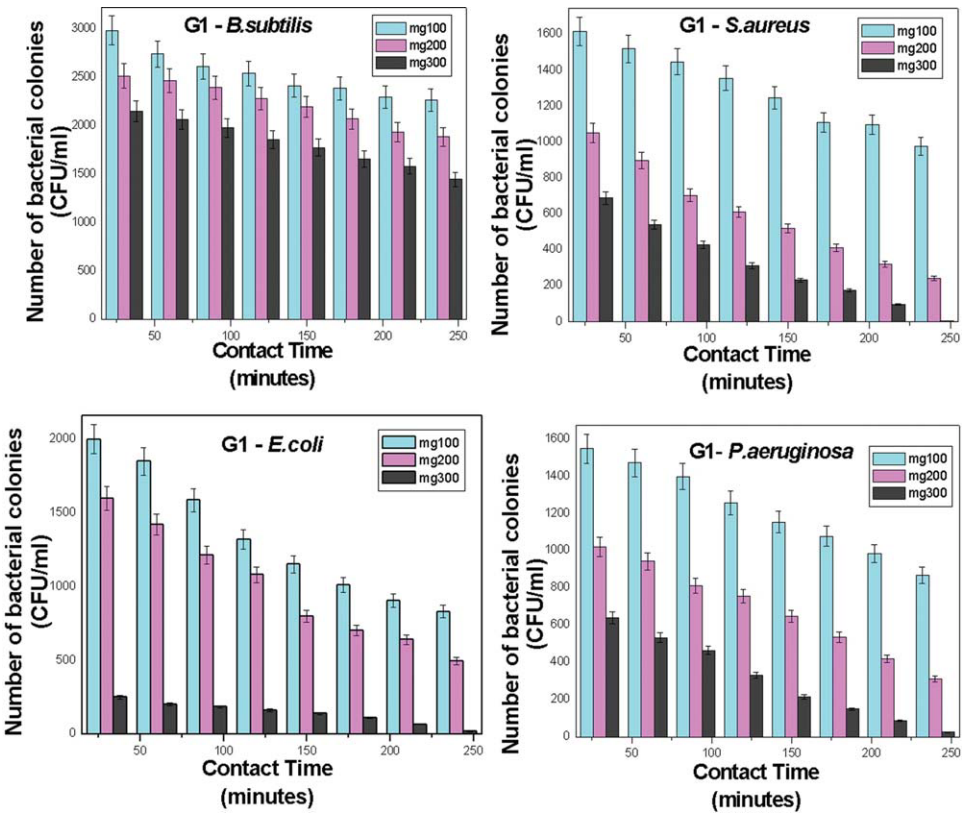

Figure 5 Graph showing the biocidal activity of G_1 copolymer beadagainst the four organisms. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

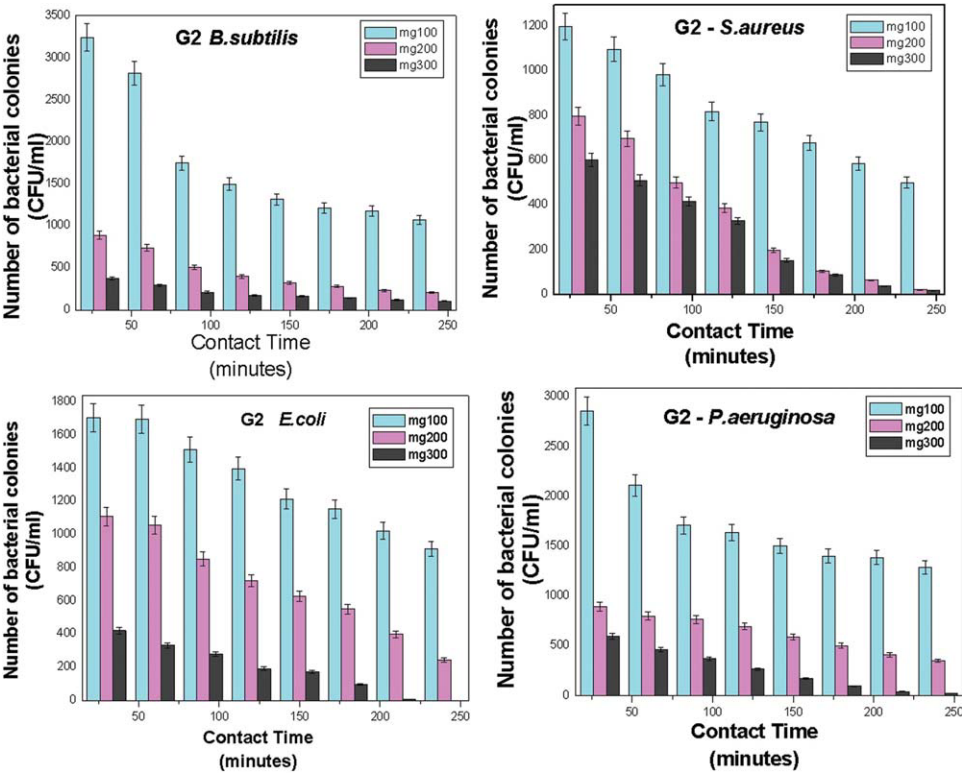

Figure 6 Graph showing the biocidal activity of G_2 copolymer bead against the four organisms. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

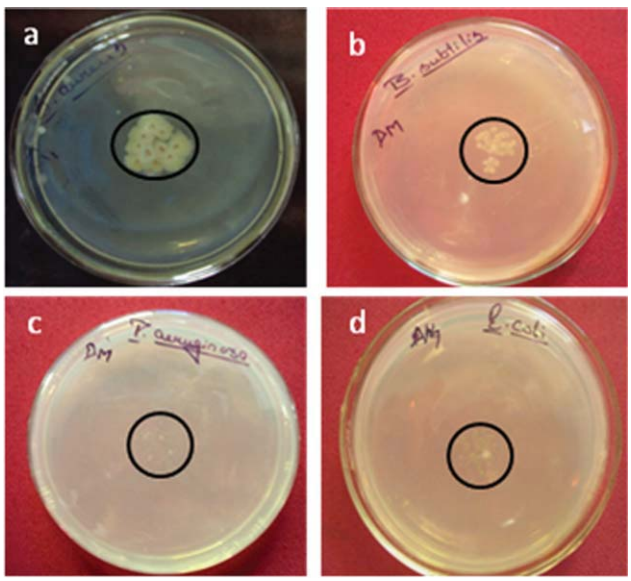

Figure 7 Test for bacterial adsorption/adhesion. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

G_1 increased. While in the case of second generation, i.e., G_2, the iterative functionalization showed even higher bacteriostatic activity compared with G_1 with increase in nitrogen content. These observations were made on the basis of the data obtained upto a contact time of 4 h with the bacterial suspension as shown in the Figures 4–6. The results therefore, suggest that the introduction of alkyl halide and amine type functionality increased the antibacterial activity by many folds.

Generally covalently bound chlorides have been less investigated for their antibacterial activity. In this study, solid supported dendritic-like architecture containing chloroethylamine type functionality has been showing better antibacterial activity. The antibacterial activities of these functional copolymer beads with the bacteria may be due to the electrostatic interactions which make them adhere to the copolymer beads. The bacterial adhesion/adsorption test conducted showed the bacterial adhesion on the copolymer beads. This adhesion/adsorption may be due to the physical entanglement, covalent bonding, hydrophobic interactions, and hydrogen bonding.[25] These forces are likely to be important in maintaining the multicellular structures that allow bacteria to cooperate metabolically and to withstand antimicrobial challenges.[26] The process of bacterial adhesion includes an initial physicochemical interaction phase and a late molecular and cellular one. It is a complicated process influenced by many factors, including the bacterial properties, the material surface characteristics, the environmental factors, such as the presence of serum proteins and the associated flow conditions.[27]

Testing bacterial adhesion/adsorption on the dendrimer type resin

After 4 h of equilibration of the dendrimers with the bacterial suspension in the test tube test method, the highly functionalized dendritic sample G_2 was used for this test. The equilibrated G_2 beads were taken out and washed with sterilized saline. The moisture in the beads was sucked with a sterilized filter paper. The dendritic bead samples were placed on the nutrient agar plates at 37°C for 24 h. The colony appearing on the plates showed adherence of bacterial cells on the copolymer bead surface. The adhesion of bacteria on the dendritic polymer surface shows that dendrimer beads exhibit bacteriostatic activity. Figure 7 shows the adhesion/adsorption of bacterial cells on the tested dendrimer type resin.

CONCLUSIONS

This investigation of solid supported dendritic architecture has thrown a new insight for using such polymers for antimicrobial application. The antimicrobial activity rendered against both Gram-positive and Gram-negative organisms might be due to the amine as well as halide functionality on the copolymer beads. The increase in the generation of dendritic functionality increased the antibacterial activity of the copolymer beads. The antibacterial activity of the synthesized dendritic architecture was found to be irreversible due to the adhesion/adsorption of the bacterial cells on the beads.

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