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Amidoxime Copolymer Beads Containing Cu/Cu_2O Microparticles as a Biocidal Material for Water Disinfection

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ABSTRACT: Macroporous crosslinked acrylonitrile-divinylbenzene copolymer beads were synthesized by suspension polymerization technique. The beads were chemically modified to have amidoxime functional group, which was used as a solid support for anchoring copper microparticles. The copper ions loaded on the copolymer beads were reduced using strong reducing agent to have copper microparticles on the amidoxime copolymer beads. The size of copper particles formed depends upon the amount of copper ions loaded on the beads. The formation of copper microparticles on the copolymer was confirmed by instrumental analysis. The copper containing amidoxime copolymer beads were investigated for the biocidal activity. The size and the distribution of copper particles on the amidoxime copolymer beads influenced their biocidal activity. The biocidal activity was tested against two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and against two Gram-negative bacteria, *Pseudomonas aeruginosa* and *Escherichia coli*. The beads containing copper particles showed better biocidal activity against the Gram-negative bacteria when compared with the Gram-positive organisms. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: copolymer; amidoxime; copper particles; disinfection; biocidal; bacteria

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INTRODUCTION

Copper has been used for biocidal application from ancient times. The biocidal activity of copper widens its usage as water purifier, algaecide, fungicide, nematocide, molluscide, antibacterial, and antifouling agents.[1] Microbial contamination of water poses a great threat to the public health as water is an integral part of human life. There is a need for new disinfecting agents as microorganisms tend to develop resistance against multiple antimicrobial agents.[2] To sort out such issues, researchers have studied the use of silver and copper ions as disinfecting agents for water.[3,4] Earlier researchers have reported the application of copper nanoparticles[5] and copper oxide nanoparticles[6] for their biocidal activities. Recently, researchers reported potential activity of copper oxide particles against pathogenic bacteria.[7] The main interest of our work was to use polymers as a stable solid support for anchoring copper particles and investigating their application in water purification. In the past decade, polymers containing nanoparticles have been investigated for their wide range of applications like in catalysis,[8] as sensors,[9] and in water purification.[10] The salient features of polymeric microspheres are their insolubility and stability, which make them a suitable candidate for anchoring metal particles. In our earlier study, we reported polymeric microspheres containing silver nanoparticles for disinfection of water.[11] The smaller particle size of copper bound onto the copolymer beads render its close interaction with the microbial membranes[12] proving them to be better biocidal material.

The amidoxime functional groups containing copolymer beads have been extensively studied for removal of heavy metal ions like Cu(II), Ni(II), Pb(II), Co(II), Cd(II), Ti(II), Mn(II), and Zinc(II) from the water bodies.[13,14] In past, researchers have reported the application potential of amidoxime copolymers containing copper and silver ions for their biocidal activity.[15] The antibacterial activity tested by plate method showed some good results against the organisms studied. The chelating ability of amidoxime functional copolymer beads to form a stable complex with the copper ions and followed by subsequent chemical reduction produces copper particles on the copolymer beads.

The continual emergence of resistant strains of bacteria demand the need for a general disinfectant that can render an active

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biocidal action against the most common bacteria present in water. Bacteria are broadly categorized as Gram-positive and Gram-negative organisms depending upon the differences in the composition of their cell walls. The Gram-positive cell wall is primarily made up of peptidoglycan, which is a polymer of *N*-acetylglucosamine and *N*-acetylmuramic acid. This has mainly carboxyl, amide, and hydroxyl functional groups. While the cell walls of Gram-negative bacteria are more complex due to the presence of an outer membrane in addition to a thin peptidoglycan layer. This outer membrane contains phospholipids, lipoproteins, lipopolysaccharides, and proteins.[16] This basic difference in the nature of the cell boundaries is a great challenge for having a general biocidal material against the bacteria.

The biocidal polymers developed were tested against two Gram-positive and two Gram-negative bacteria to study the antibacterial activities of newly prepared polymer supported copper microparticles. *Escherichia coli* ATCC 8739 (NCIM 2065) was selected as a fecal contamination indicator, where as *Pseudomonas aeruginosa* ATCC 9027 (NCIM 2200) as a representative of soil contamination indicator and *Bacillus subtilis* ATCC 6051 (NCIM 2920) and *Staphylococcus aureus* ATCC 25923 (NCIM 5021) as indicators of human influence in water bodies were used.

MATERIALS AND METHODS

Acrylonitrile and toluene were purchased from S.D. Fine chemicals (India). Divinylbenzene was purchased from Aldrich (Bengaluru, Karnataka) and benzoyl peroxide was obtained from Heny fine chem (India). Cupric chloride, hydrazine hydrate, hydroxylamine hydrochloride, sodium hydroxide, and methanol were all procured from Finar Chemicals Limited (India). Nutrient Broth and Nutrient agar used for the growth and maintenance of bacterial cultures were purchased from Hi-Media Laboratories (Mumbai, India).

Bacterial Strains

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). The 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 24-h-old.

Synthesis of Macroporous Amidoxime Functional Copolymer Beads

Acrylonitrile crosslinked copolymer was synthesized by following reported suspension polymerization technique.[17] Monomer mixture containing acrylonitrile, divinylbenzene, toluene as a porosogenic agent, and benzoyl peroxide (1% w/w) was prepared separately in required proportion and added to the preheated suspension medium maintained at 70°C in a three-neck round-bottom flask. The entire reaction mass was gradually heated to 80°C under constant stirring and reflux conditions for further 5 h. During this period, the copolymer beads separated out in the form of spherical opaque beads. The acrylonitrile copolymer beads were further chemically modified using hydroxylamine hydrochloride to have amidoxime functional groups onto the copolymer matrix.[18] The product obtained was then cooled and washed thoroughly with hot water. The functional group conversion was confirmed using fourier transform infrared spectroscopy spectral studies provided in the Supporting Information.

Synthesis of Cu/Cu_2O Microparticles Bound Amidoxime Functional Copolymer Beads

Amidoxime copolymer beads (4 g) were equilibrated with 0.025, 0.05, and 0.075 *M* $CuCl_2$ solution separately for 16 h at room temperature. The copper loaded resin beads were thoroughly washed with distilled water to remove excess of $CuCl_2$. The amount of copper loaded on the beads is given in Supporting Information Table 1; afterward the beads were kept in 40 mL of 1 *M* NaOH separately for 2 h. The beads were then washed with distilled water to remove excess alkali and kept in 40 mL of 2:1 ratio of hydrazine hydrate and water for 60 min at 80°C. The images of copper particles bound amidoxime copolymer beads of three different concentrations are shown in the Supporting Information (Figure 1). The amidoxime copolymer beads equilibrated with 0.025 *M* $CuCl_2$ is named as C25. Similarly, the beads equilibrated with 0.05 and 0.075 *M* are named as C50 and C75, respectively.

Characterization of Copper Particles Bound Amidoxime Functional Copolymer Beads

The amidoxime copolymer beads containing copper particles were observed using optical microscope Olympus SZX16 for the surface variation on the copolymer beads before and after the formation of copper particles on them. The scanning electron microscope (SEM) images were observed using Leo series (VP1430), which showed the formation of copper microparticles on the copolymer beads. energy-dispersive X-ray spectroscopy (EDX) analysis further confirmed the loading of copper on the amidoxime copolymer beads. Powder X-ray diffraction (XRD) patterns were recorded with Philips X'Pert MPD system using Cu K_α radiation ($\lambda = 1.5406$ Å) for the copper particles bound amidoxime copolymer beads before and after the equilibration with water. The X-ray Photoelectron Spectroscopy (XPS) analysis was performed to identify the change in oxidation state of copper on the copolymer beads.

Biocidal Experiments

This study was undertaken to investigate the antibacterial efficacy of the copolymer containing copper particles against both the Gram-positive and Gram-negative model organisms.

Plate Method. Nutrient agar was poured into the plates and allowed to solidify. A 100-μL of 24-h-old undiluted bacterial culture was spread on separate plates uniformly. The dry copolymer beads containing copper particles were placed over the solidified nutrient agar gel in the Petri plates. The copolymer without copper particles was used as blank. These plates were then subjected to incubation for 24 h at 37°C.

Test Tube Test (Batch Method). The antibacterial activity of the copolymer beads containing copper particles was tested against selected Gram-positive and Gram-negative bacteria by test tube method. A 100-μL of each bacterial culture were added to the test tubes containing 20 mL of autoclaved water separately. Copolymer beads of 100 mg were added into each of the test tubes containing four test cultures to investigate the reduction in bacteria count as a function of time. The tubes were then kept on the rotary shaker platform at 120 rpm. Similarly, experiments were conducted by taking 200 and 300 mg of copolymer beads. This variation was done to optimize the quantity of the copolymer beads required for complete inhibition of the bacteria. Fractions of 100 μL were withdrawn after every hour and upto 6 h to find

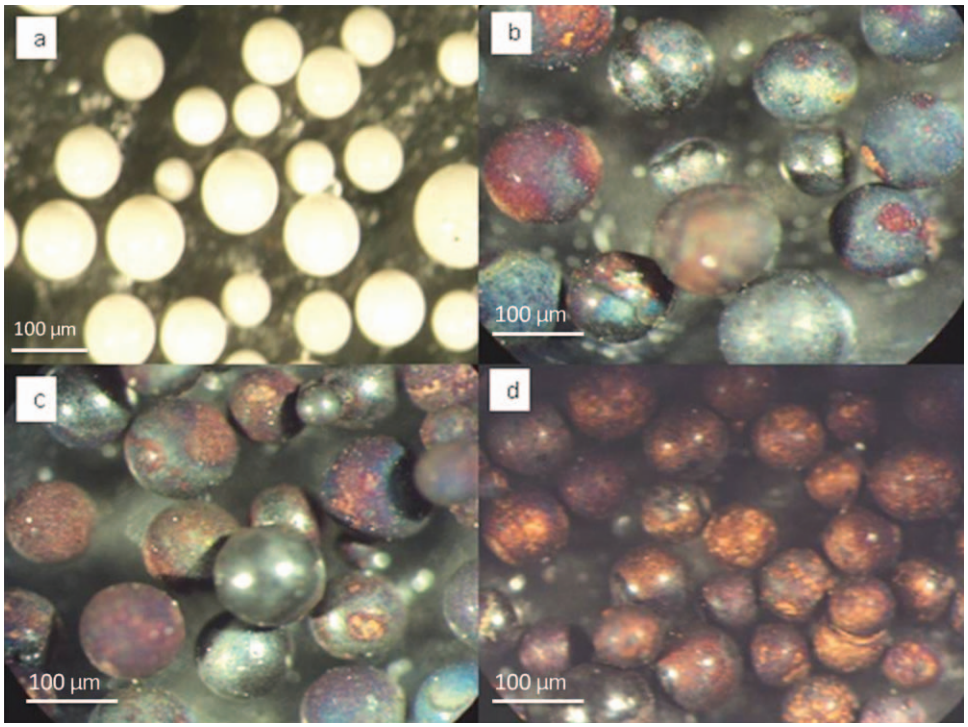

Figure 1. Optical microscope images of (a) amidoxime copolymer beads. Amidoxime copolymer beads containing copper microparticles (b) Cu 25, (c) Cu50, and (d) Cu75. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

out the reduction in the bacterial count. The fractions were then plated after single dilution to obtain the bacterial colony count. The copolymer beads without copper particles were used as blank in the experiment. The initial bacterial count was in the range of 16 to 137 $\times$ 10^6 colony-forming unit (CFU)/mL.

RESULTS AND DISCUSSION

The copper particles bound copolymer beads were subjected to various analyses showed the formation of copper particles on the copolymer beads and their interactions with the bacterial cell rendering the biocidal activity.

Optical Microscope

The copper particles bound copolymer beads were observed under optical microscope for any cracks or ruptures on the surface of the beads. Optical microscopic observation revealed no such damage to the copolymer beads during chemical reduction. The formation of copper microparticles was observed, as the beads turned brown black. The beads containing higher copper content had metallic luster (Figure 1). The metallic copper being unstable in air tends to turn black with the passage of air contact.

The copper particles loaded beads were kept in contact with water to check their stability for water disinfection purpose as shown in Figure 2. The Cu25 beads turned pale green, while Cu50 beads showed deep green, and Cu75 had brownish-green when kept in water. The change of color of the copolymer beads containing copper particles was due to the formation of cuprous oxide on the bead surface due to the air oxidation.

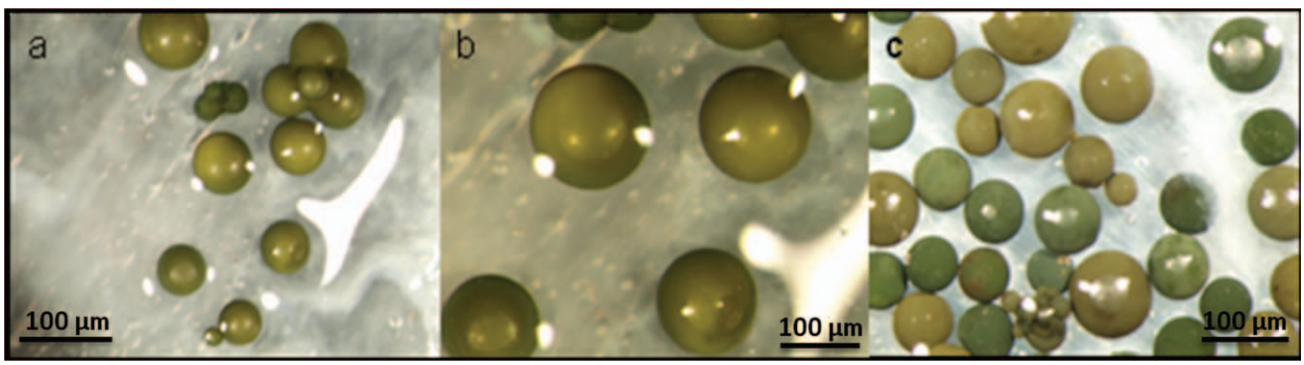

Figure 2. Optical microscope images of copper microparticles bound amidoxime copolymer beads kept in water; (a) Cu25, (b) Cu50, and (c) Cu75. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

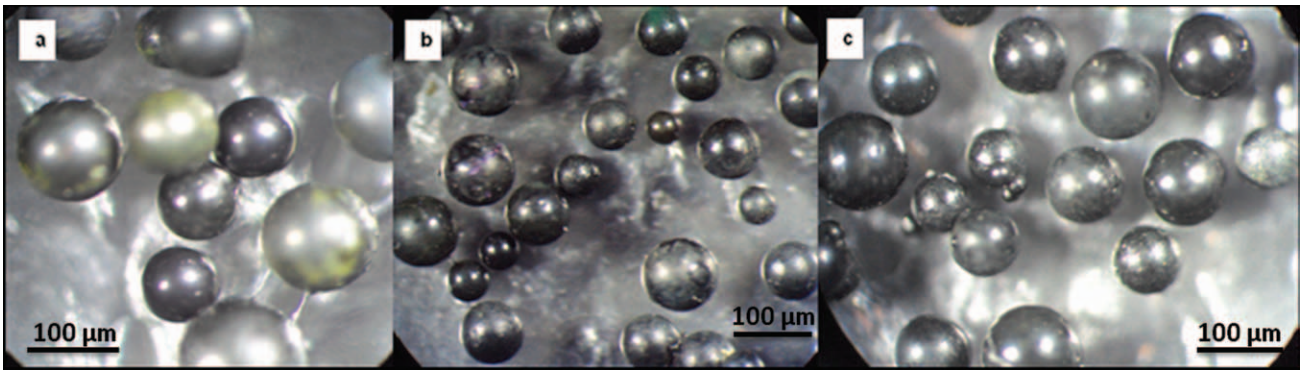

Figure 3. Optical microscope images of Copper microparticles loaded amidoxime copolymer beads dried after water contact; (a) Cu25, (b) Cu50, and (c) Cu75. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The formation of hydrated copper oxide showed green color in the wet condition. When the same beads were dried in air, they turned black showing the formation of cuprous oxide. The formation of cuprous oxide is shown experimentally in the Supporting Information. The color change of copper particles with change in medium was also reported by Ref. 19. All the three samples turned brown black after their removal from the water and subsequent drying in air as shown in Figure 3. This change was observed within 5 min of contact time with air and remained as such thereafter. Similarly, researchers reported the stability of copper nanoparticles in water which is synthesized by arc fabrication method.[20]

SEM/EDX Analysis

The surface morphology of copolymer beads containing copper particles was examined by SEM, and their elemental composition was analyzed using EDX spectra. The copolymer beads containing copper particles show variation in the particle size with an increase in the copper concentration as given in the Figure 4. The copper loading on the copolymer beads increased with increase in copper ions concentration resulting in agglomeration of copper particles.

An EDX machine directly attached to the SEM instrument indicates the existence of C, O, N, and Cu elements on the resin beads. This correlates with the structure of amidoxime functional copolymer beads. The EDX spectra of this copolymer are provided in the Supporting Information.

Powder XRD Analysis

The powder XRD patterns in Figure 5 shows distinct peaks for copper particles on the amidoxime functional copolymer beads. The [111] plane of Cu25, Cu50, and Cu75 shows 2θ values as 43.38, 43.41, and 43.61, respectively. The 2θ values 50.48, 50.61, and 50.71 correspond to the [200] plane, whereas, for [220] plane the values obtained are 74.03, 74.35, and 74.44, respectively. All these [111], [200], and [220] planes correspond to Cu(0) (JCPDS No.85-1326) microparticles on the copolymer beads.[21] The formation of cuprous oxide on the bead surface is below detection limit as the conversion of copper to cuprous oxide may be less on the copolymer beads.

Powder XRD Analysis After Water Equilibration

To study the changes after the interaction of copper particles containing copolymer beads with water, the powder XRD patterns was obtained after equilibrating them in the water as shown in Figure 5. The water interaction of the copolymer beads decreases the [111] plane peak intensity of Cu25 when compared with that of Cu50 and Cu75. This is attributed to

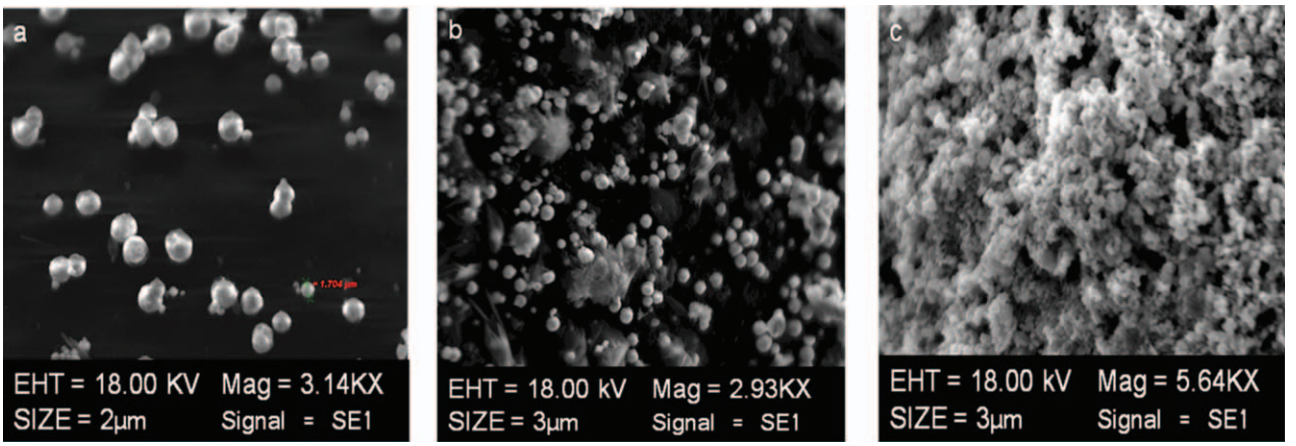

Figure 4. SEM images of amidoxime copolymer beads containing copper particles. (a) Cu25, (b) Cu50, and (c) Cu75. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

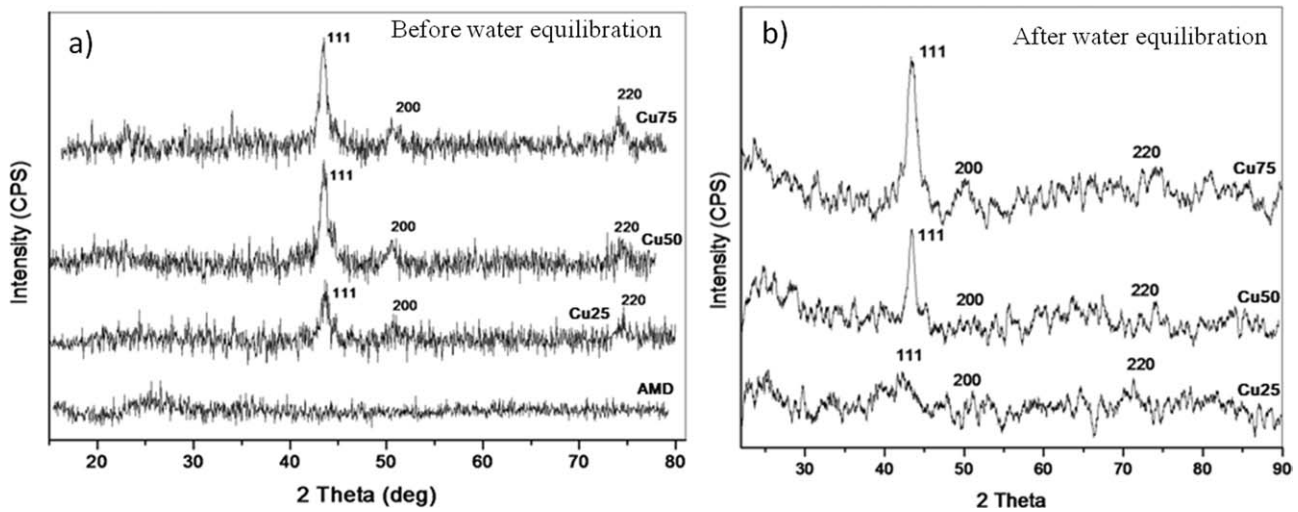

Figure 5. Powder XRD pattern amidoxime copolymer beads containing copper microparticles (a) before water equilibration and (b) after water equilibration.

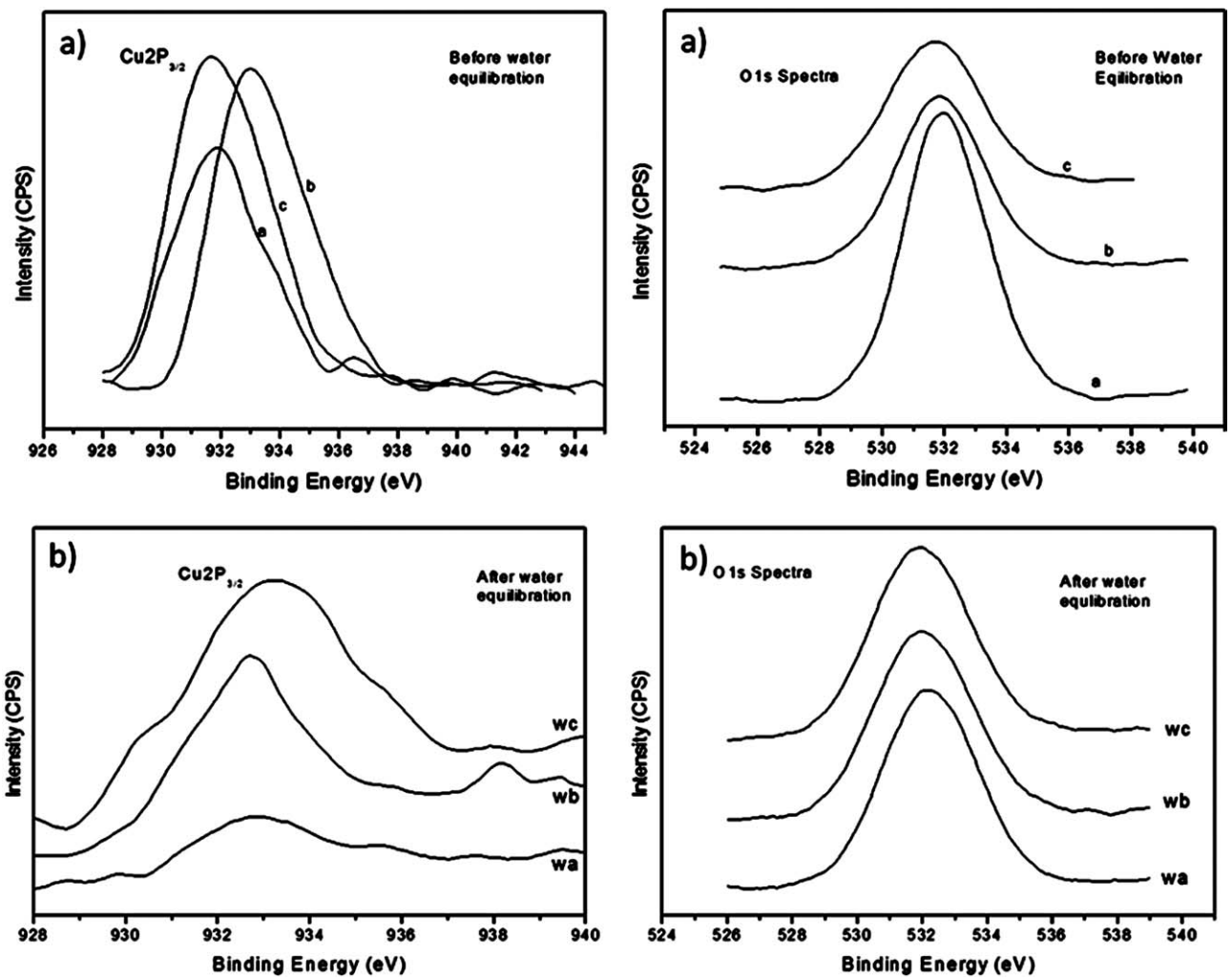

Figure 6. XPS analysis of copper microparticles bound amidoxime functional copolymer beads (a) before water equilibration and (b) after water equilibration.

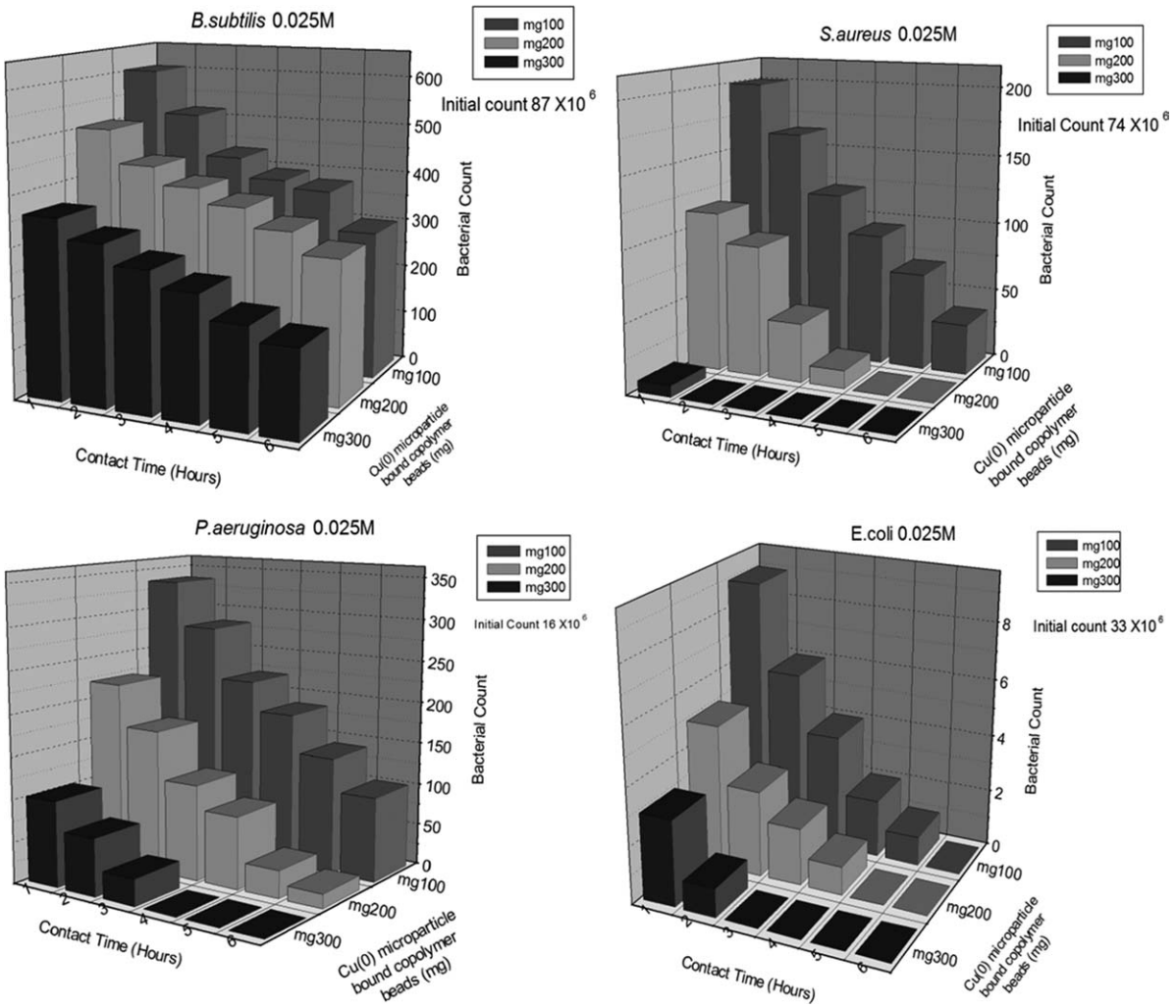

Figure 7. Graph showing the biocidal activity against the four organisms tested for Cu25.

more interaction of water with copper particles at lower concentration.

The lower surface change observed on the copolymer beads with higher copper loading shows the distinct [111] plane peak with same intensity even after water equilibration. This indicates that in case of higher concentration of copper loading agglomeration prevents water interaction with the copper particles.

XPS Analysis

XPS analysis was performed for all the three copper loaded polymers to study the change in oxidation state of copper on the copolymer beads. Results given in Figure 6 shows that the majority of particles on the copolymer beads are of Cu(0). Powder XRD analysis also supported the similar results. The formation of cuprous oxide on the surface of the copolymer beads was not detected distinctly, as the electron volts energy difference is less to distinguish between copper and cuprous oxide. It proves that the majority of copper particles formed on the copolymer beads are stabilized by the amidoxime functional group of the copolymer network. O1s XPS spectra values observed before and after water equilibration was in the range of 531.7–532.0 eV, which corresponds to the oxygen atom of the amidoxime functional group of the copolymer beads. The O1s binding energy values above 531.7 eV suggest the absence of copper oxide on the copolymer beads.[22] Satellite peak of $Cu2P_{3/2}$ was not observed in the XPS analysis, which confirms the predominant formation of Cu(0) particles on the copolymer beads, and there is no significant change taking place in $Cu2P_{3/2}$ peak even after water equilibration.

BIOCIDAL STUDIES

The biocidal activity of the copper particles bound amidoxime beads was tested by plate and test tube method.

Plate Method

A preliminary experiment was conducted with copper particles bound copolymer beads Cu25, Cu50, and Cu75. The zones of inhibition was measured for all the four bacterial cultures and the values of each are given in Supporting Information Table 2. Copper particles prepared from all the three different concentrations exhibit good antibacterial activity against all the four selected bacterial strains.

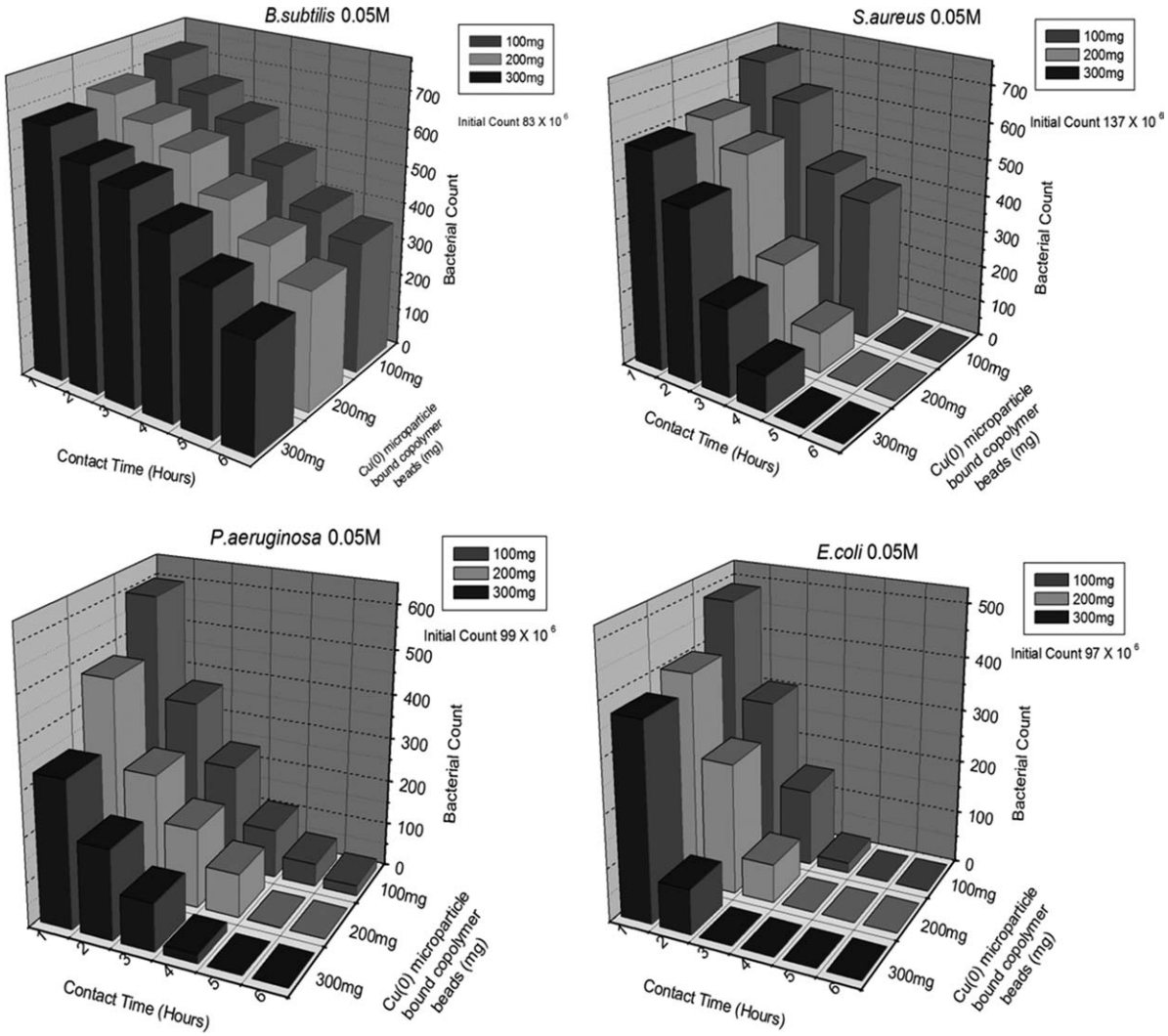

Figure 8. Graph showing the biocidal activity against the four organisms tested for Cu50.

Test Tube Method

The sample Cu25 reduces the bacterial count to zero with an increase in contact time, except for *B. subtilis*. Viable cells of *S. aureus* were reduced to zero within 2 h of contact time with 300 mg of copper particles bound copolymer beads, while in the case of Gram-negative bacteria *E. coli*, it reaches nil after 3 h of contact time owing to their outer cell wall complexity. In case of *P. aeruginosa*, for 300 mg of copper particles bound copolymer beads, the count becomes zero after 4 h of contact time. This difference in bacterial reduction among the Gram-negative organisms is due to the higher permeable nature of *E. coli* cell wall.[23] Increase in antibacterial activity is noted with an increase in the quantity of copper particles bound copolymer beads. Among the Gram-positive organisms tested, complete inhibition within 2 h of contact time was achieved for *S. aureus*, while *B. subtilis* being a spore forming organism shows resistance against complete inhibition (Figure 7).

Cu50 sample requires more contact time for attaining bacterial inhibition when compared with that of Cu25 beads shown in Figure 8. For 300 mg of copper particles bound Cu50 copolymer beads, it took 5 h for complete inhibition in case of *S. aureus*, while it was 3 h for *E. coli*, and 5 h for *P. aeruginosa*. Except *B. subtilis*, all the other cultures tested show zero bacterial count. In case of 100 and 200 mg of Cu50, complete bacterial reduction needs more contact time compared with 300 mg of copper particles bound copolymer.

Cu75 copolymer beads shows less biocidal activity when compared with Cu50 and Cu25 beads. This may be due to the high agglomeration of copper particles on the copolymer at a higher concentration of $CuCl_2$. This is also supported by SEM images. Zero bacterial count was achieved for 300 mg of copper particles bound copolymer beads only in the case of Gram-negative bacteria. This shows that Gram-negative species are prone to copper particles than Gram-positive bacteria owing to the difference in the cell wall composition. The activity of the Cu75 beads is shown in the Figure 9. Due to the agglomeration of copper particles on the copolymer beads, their biocidal performance decreases. The higher permeability of outer cell wall in *E. coli* shows complete inhibition for all the three concentrations of copper particles.

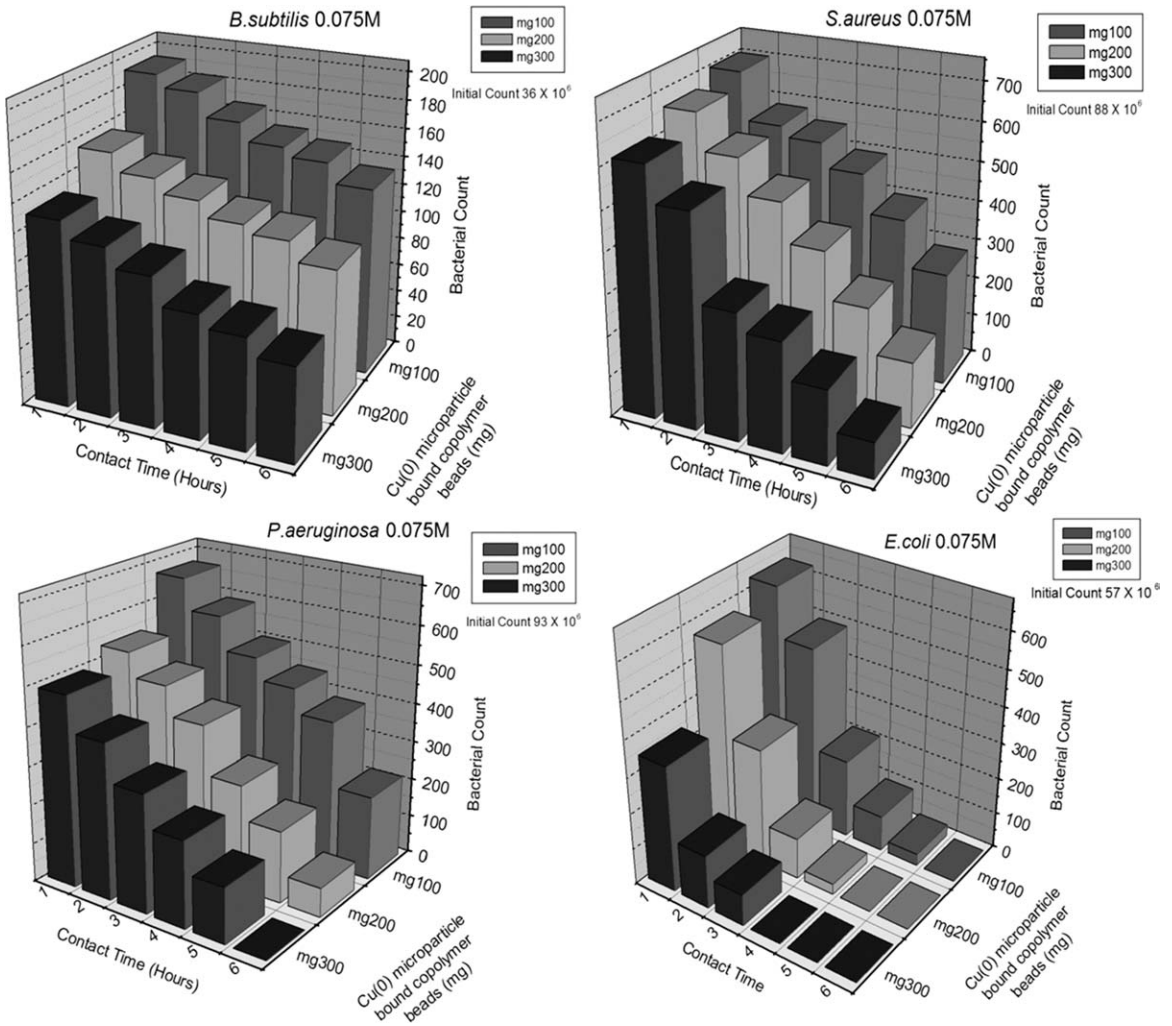

Figure 9. Graph showing the biocidal activity against the four organisms tested for Cu75.

As the quantity of copolymer beads increases from 100 to 300 mg, the biocidal activity against the four organisms tested increases. But with an increase in the concentration from 0.025 to 0.075 *M*, the biocidal activity decreases. This property can be attributed due to the agglomeration observed with an increase in the concentration of copper particles loaded on the copolymer beads, which is shown in SEM analysis.

CONCLUSION

The amidoxime functional group renders a stable solid support for the formation of copper particles on the copolymer beads. The stability of the copolymer beads throughout the chemical process is due to their higher percentage of crosslinking. The copper loading at lower concentration gives smaller particle size of copper which renders effective biocidal activity. The leaching of copper particles in the water is prevented by the chelating ability of amidoxime functional group. As the contact time is prolonged, complete inhibition was achieved for all the other bacteria tested except for the spore forming *B. subtilis*. This promises amidoxime functional group containing copper particles as a suitable material for water disinfection.

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