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MORPHOSTRUCTURAL CHANGES INDUCED IN E. COLI EXPOSED TO COPPER IONS IN WATER AT INCREASING CONCENTRATIONS

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ABSTRACT

Atomic Force Microscopy (AFM) has been used to elucidate the morphostructural changes occurring as a result of copper internalization in *E. coli*. The study documents a gradual aqueous leaching and built-up of copper ions within the cell using Atomic Absorption Spectroscopy (AAS). It further correlates the same to the morphometric changes studied using AFM and the cell viability studied using confocal microscopy. The cell perturbations take place within one hour of exposure to copper ions in water. Cell elongation takes place with the increase in exposure time and therefore copper ion concentration. Partially perturbed structure of the cell and accumulation of the cytosolic contents towards the apical ends of the cell reveal the inside-out mode of cell damage caused by copper ions.

KEYWORD: *E. coli*, copper, Atomic Force Microscopy, Confocal Microscopy, Atomic Absorption Spectroscopy, Morphostructure.

Abbrevations: AAS- Atomic Absorption Spectroscopy; AFM- Atomic Force Microscopy.

1. INTRODUCTION

Bacterial cell integrity and morphology gets influenced by the environment in which the cells exist. Plate assay technique is conventionally used to study these changes in a large population of cells (Torrent et al., 2010). Single cell microbiology has however enabled researchers to study the cell alterations in response to these environmental changes. Flow

cytometry, micro-spectroscopic methods and Atomic Force Microscopy (AFM) are a few examples of single cell microbiological techniques (Torrent et al., 2010). AFM however remains a powerful imaging tool for the multi-parametric analysis of the microbial cells providing insights into the detailed structure-function relationship (Drake et al., 1989; Engel and Muller, 2000; Müller and Dufrêne, 2008). As compared to other microscopic techniques, AFM enables imaging cellular structures at molecular resolution without the need of coating, staining or damaging the biological sample (Alessandrini and Facci, 2005; Müller and Dufrene, 2008; Franz and Puech, 2008; Amiji et al., 2008). The potential of AFM has been employed to monitor the cell wall response to the stress induced by β-lactam group of antibiotics like cefodizime, penicillin, amoxycillin, rokitamycin (Braga and Ricci, 1998; 2000; Yang et al., 2006). AFM studies to evaluate the effects of peptides (Meincken et al., 2005; Ramamoorthy et al., 2005; Alves et al., 2010) on microorganisms has been well documented. AFM studies of morphostructural modifications induced by carbon, silver and copper nanoparticles have also been reported. Copper has been known to exhibit antimicrobial effects since ancient times and the studies carried out by Tandon et al. (2005) have effectively demonstrated the bactericidal effect of copper and its alloys on a large population of cells. Espirito Santo et al. (2011) also have reported on the ability of copper to affect the cell membrane-DNA as well as membrane-proteins.

The present study was designed to investigate structural changes induced by copper ions using AFM, a single cell microbiological technique wherein morphostructural alterations like height of the cell, elongation, surface corrugation have been measured. The amount of copper leached in the aqueous media and internalized by the cells has been estimated using AAS. These measurements correlated with the capacity of the organism to revive in the nutrient medium using confocal microscopy. The use of transition metals like silver and copper as effective antibacterial has been reported in the literature (Feng et al., 2000, Landsdown, 2010, Shrestha et al., 2009). The damage of bacterial cell and subsequently its death caused due to the exposure to silver ions as reported by Yang et al. (2010) was predominantly due to puncturing of holes in its cell wall. Our results with copper however paint a different picture of cell damage and death. The study succeeds in associating copper internalization to the bacterial cell structure collapse. The present work for the first time, to the best of our knowledge, gives an evidence using AFM of an inside-out mode of cell damage caused by copper ions in water, which is different than silver ion induced effects reported in the literature (Yang et al., 2010).

2. MATERIALS AND METHODS

Copper sheet was incubated in sterile deionized water for a period of 6 h. This resulted in increasing concentration of Cu ions leached in water. 10^6 CFU/ml of *E. coli*, gram negative bacteria were exposed to the increasing concentration of copper ions (assessed using AAS) as a function of time (1-6 h). Bacteriostatic activity was assessed using microbiological assays/plating with exposure to copper ions for different time periods (1-6 h). Morphostructural changes were studied using AFM and the cell viability was studied using confocal microscopy for *E. coli* untreated (Control) and exposed to copper ions leached in water for 1-6 h. The following aspects are elaborated in the following section.

2.1. Determination of copper concentration in deionized water

All glasswares were submerged overnight in 10 % nitric acid to remove any contaminating metal ion residues. A copper sheet (purity 99.70 %) with the surface area of 96 cm^2 procured from Hi-Media (India), was washed with distilled water several times and used to cover the base of a glass beaker containing 50 ml of deionized water. A series of such aqueous systems were maintained for 6 h on an orbital shaker (200 rev min^{-1}) at room temperature (28 ± 2 °C) for estimation of the copper concentration using atomic absorption spectroscopy (AAS). At different time intervals, water samples from the systems were subjected to acid digestion (nitric acid: HCl- 1:3) (Geldreich, 1975), reconstituted with 50 ml deionized water and analyzed using Perkin Elmer A Analyst 400 Massachusetts, USA.

2.2. Bacterial culture preparation and kill kinetics for E. coli

Wild type *Escherichia coli* culture was grown to log phase in Luria Bertani broth at 37 °C for 18 h. Bacterial concentrations were determined by measuring the absorbance of the culture at 600 nm in colorimeter (0.1 - 10^8 cells/ml). Stock cultures of *E. coli* were maintained at -20 °C. An aliquot of 18 h old culture of wild type *E. coli* was added to the aqueous system containing copper sheet (purity 99.70 %, surface area = 96 cm^2) to achieve a concentration of 10^6 cells/ml. A series of systems were maintained at room temperature (28 ± 2 °C) with constant shaking at 200 rev min^{-1}. At every time interval the cells were harvested from the system centrifuged and washed with deionized water and subjected to acid digestion for analysis of copper internalized by the cells using AAS. From parallel set of systems cells were harvested to study the viability by plating on LB agar plates as well as suspending them in LB broth. The samples were also processed simultaneously for confocal microscopic analysis. The plates and broth, incubated at 37 °C, were observed for growth at the end of 24

h. Control systems without copper sheets were maintained, harvested and subjected to similar treatment and analysis during the study.

2.3. Confocal laser scanning microscopy

An aliquot of 1 ml was drawn from each of the copper system centrifuged at 4000 rpm for 10 min. The bacterial cells were subsequently washed using de-ionized water to remove free ions or copper ions adsorbed on the cell wall, if any, and then resuspended in 100 µl deionized water. Differential staining of these bacterial cells was carried out using Acridine Orange (AO) and Ethidium Bromide (EB) (Sigma-Aldrich, India) (Kasibhatla et al., 2006). The samples were imaged using NIKON A1R-A1 confocal microscope system equipped with a ECLIPSE Ti-E inverted microscope, 60 x oil immersion objective lens, and Multi-Ar (457/488/514 laser). A 510 long pass primary dichroic mirror was used for excitation of EB and AO. Fluorescence of AO monomers (490-550 nm) and luminescence of AO aggregates (640 - 700 nm) were detected using a 522/30 band pass and 640 long pass filter, respectively. The diameters of the pinholes were 2.0 mm (2.4 Airy units) and 1.9 mm (1.8 Airy units) in the green and the red bands, respectively. The images were analyzed using Nikon NIS-Elements software. One image was a sum of 20 consecutive scans (3.5 s each). The size of a pixel was 0.338 µm (zoom 1.0) unless otherwise stated. The average pixel dwell time was 13 µs. Fluorescence was detected using photomultipliers in photon-counting mode.

2.4. Atomic force microscopy

To understand the morphometric alterations due to copper ion internalization, the cells were subjected to AFM analysis. The bacterial smears were air dried and observed immediately so as to obtain unaltered topographical images. To monitor the effect of copper leaching on *E. coli*, 10 µl of sample from each of these flasks including the control were drawn and placed on an ultrasonically cleaned microscopic slide, air dried at 25 ^{o}C for 20 min, washed and used for imaging. AFM images were recorded using a JPK instrument NANO wizard II, Berlin, Germany. AFM was operated in the intermittent contact mode equipped with a 100 × 100 × 15 µm XYZ closed loop scanner. The bacterial cells were scanned using silicon cantilevers with resonance frequency of 75 kHz and spring constant of 2.8 N/m. The scan speed was maintained at 0.5 to 1 Hz with 256 pixels per line scan. A topographical image (represented by height and amplitude) and a phase image were obtained simultaneously in each scan. The lighter region indicates higher height in topographical image, and more rigid (i.e. less compliant) component in phase image. Cross sections of average 5 randomly selected

individual bacterial cells from every time period (1-6 h) was obtained using the JPK image processing software, and their average is reported. The images were processed by means of first order plane fitting. Roughness measurements were carried out on five different areas of 200 × 200 nm size, and their average is reported.

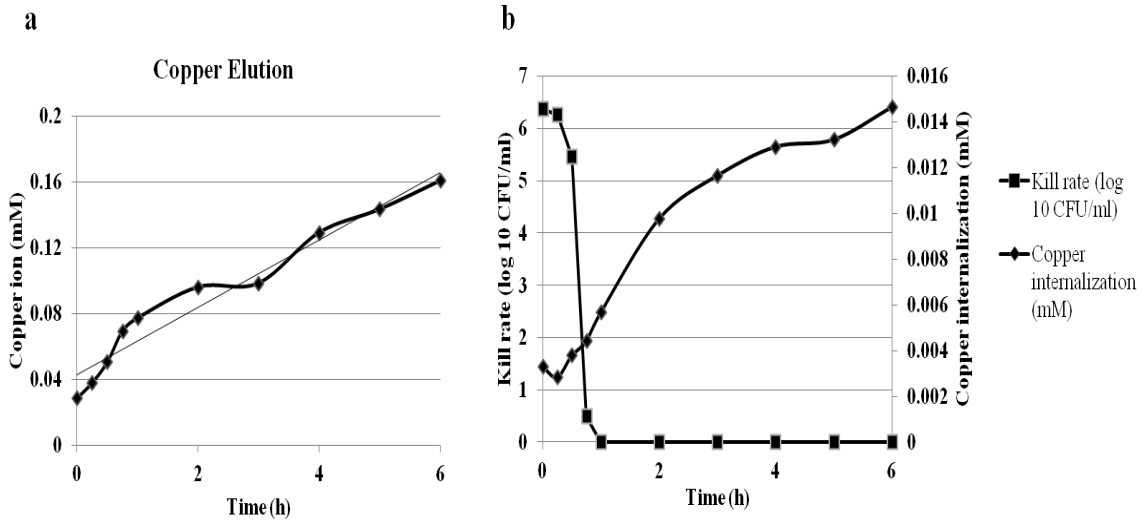
2.5. Statistical analysis

All experiments were performed in replicates for each series of measurements with respective controls. The experimental results were analyzed by one-way ANOVA using the INSTAT software. The level of statistical significance was set at $P < 0.05$ for treated cells versus untreated controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3. RESULTS

3.1. Extracellular and intracellular copper concentration using AAS

Incubation of copper sheet in sterile deionized water over a period of 6 h resulted in a gradual leaching of the metal in its surrounding aqueous medium (R^2=0.95), with maximum concentration of 0.16 mM at 6 h (Fig. 1a). Figure 1b, shows the plot of kill rate of bacteria and the simultaneous intracellular built-up of copper ions by the bacterial cells. The gradual leaching of the copper ions and their uptake by the bacterial cells is proportional to the leaching time. The copper internalization showed significant increase ($p < 0.01$) from 6.6 × 10^{-11} to 2.9 × 10^{-10} µM of copper ions/cell between 0- 6 h(Fig. 1b).



Figures

Fig. 1. a) Elution of copper ions as a function of leaching time, b) kill kinetics and copper ions internalized by the cells as a function of leaching time.

3.2. Antibacterial activity of copper leaching time –Plate and broth method

To determine the concentrations of copper and time that could induce the non-culturable conditions; mid- to late- exponential-stage cells of *E. coli* were exposed to copper in aqueous medium and enumerated on (LB) agar as well as liquid medium (broth). The gradually eluted copper exhibited static effect on the bacterial cells with no colony forming units on plates within 1 h of exposure (1.13×10^{-10} µM copper ions/cell). However, the cells could survive in the nutrient broth till 2 h (1.95×10^{-10} µM copper ions/cell) resulting in the bactericidal effect of copper, indicating the setting in non-culturable conditions of the cells within 1-2 h (Fig. 2). It is clearly evident from the graph that the ratio of the copper internalized by the cells and the bacteria killed, reached optimization at 30 min (7.6×10^{-11} µM copper ions/cell), indicating the onset of bacterial cell stress (Fig. 1b).

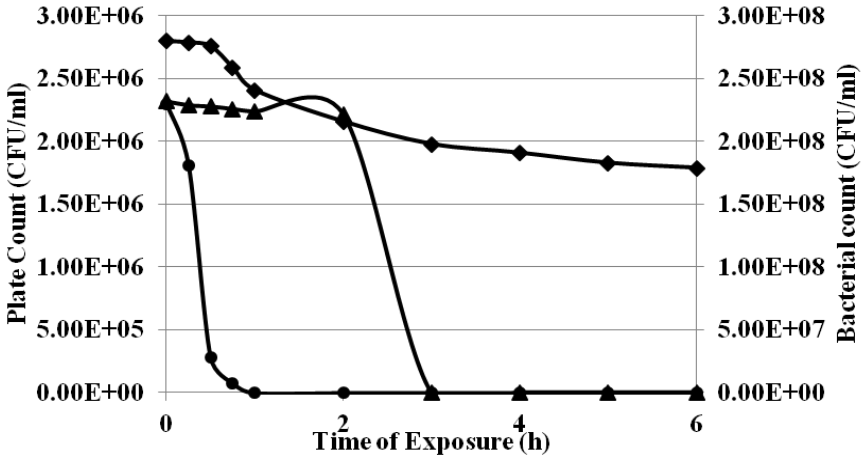


Fig. 2. Cell density as a function of time of exposure to copper ions: on plate -◆- untreated, -●- treated and -▲-in broth

3.3. Viability of bacterial cell imaging by confocal microscopy

To verify the antibacterial activity of copper ions, the bacterial cells were incubated for time intervals from 0-6 h and were assayed using live/dead (ethidium bromide/acridine orange) viability assay (Fig. 3). The two fluorescent dyes differentiate cells, from the cells with undamaged cell-membrane to the cells with compromised cell-membranes with their ability to permeate differentially. As seen from figure 3(a), the bacterial cells (control) show green staining; however on exposure with copper containing water there was an increase in the ratio of red/green fluorescence (i.e. dead: live cells) with the increase in copper concentration (Fig. 3b-f). The fluorescence intensity of AO/EB in the cells indicates significant reduction in the live cells and ascends in the number of red cells. Thus a loss of cell membrane integrity can

be seen in the bacteria exposed to copper ions in aqueous medium (Fig. 4). The survival rate of bacteria in the presence of copper ions significantly decreased from 85% to 45% ($p < 0.005$) indicating initiation of membrane damage at 2 h of exposure time.

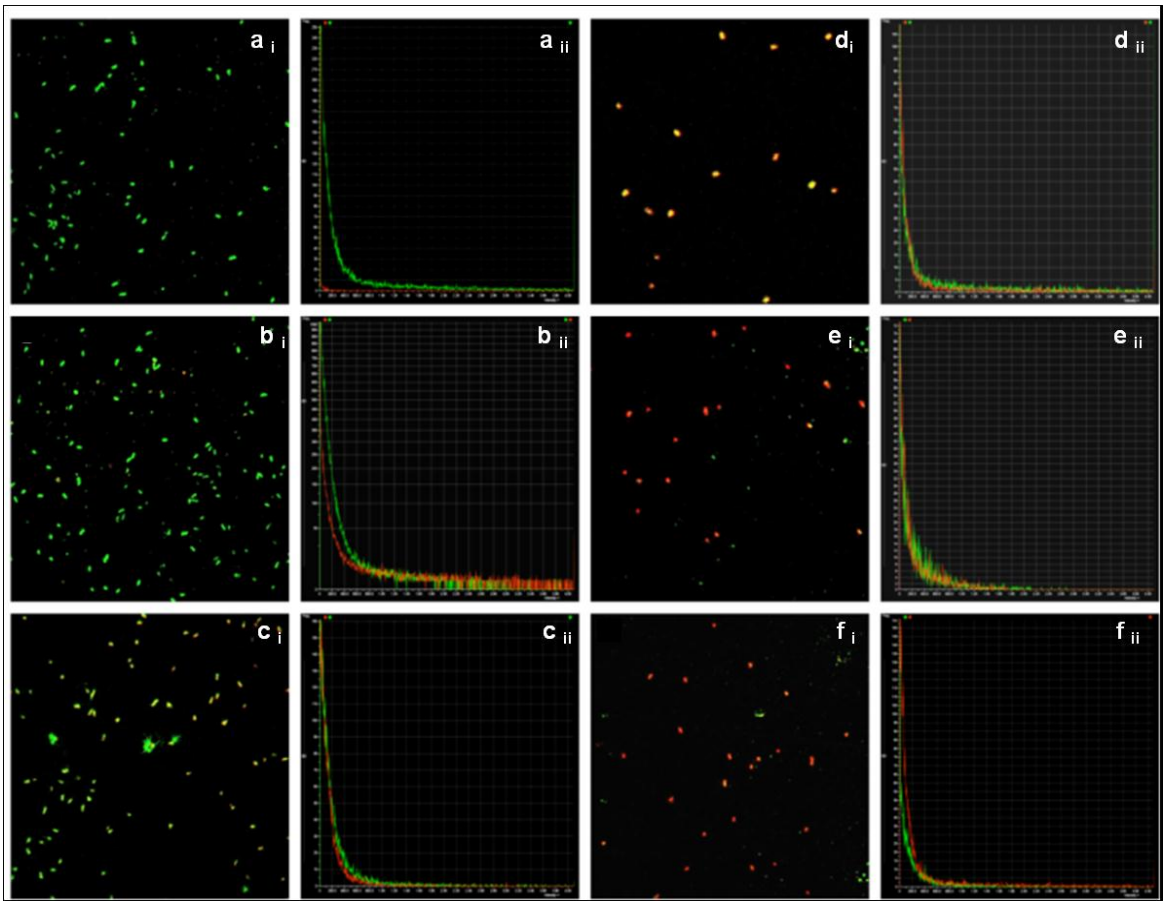


Fig. 3. (i) Confocal images 60X of *E. coli*: (a) Control (b-f) exposed to copper water for 2-6 h. (ii) Measurement of red/green intensity.

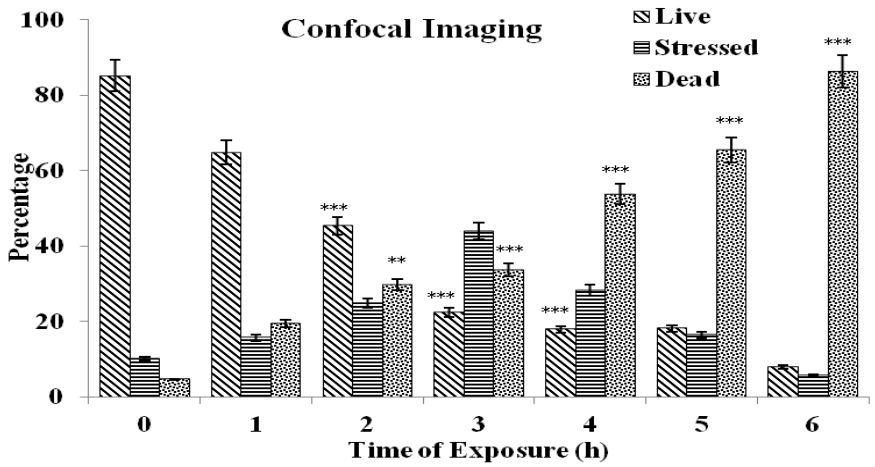


Fig. 4. Graph of response to increasing copper stress in terms of dead, live and stressed cells as a function of exposure time (- $p < 0.01$, ***- $p < 0.005$).**

3.4. Morphostructural imaging of E. coli using atomic force microscopy

The topography and amplitude images along with the phase contrast and three dimensional images of the unexposed (control) bacterium *E. coli* (Fig. 5) shows typical rod-shaped cell with relatively smooth surface without any alterations. The images of *E. coli* exposed to copper ions leached in water at different time intervals aided in revealing the gradual cell structure collapse. This is evident through the cross-section profiles (Fig. 5e) & (Fig. 6e), depicting significant decrease in the average height of the cells from 550 ± 50 nm (Fig. 5e) for control to 220 ± 20 nm (Fig. 6e) for cells exposed at 6 h, as well as increase in the length of the cells from 2.0 ± 0.5 µm (control) to 5.0 ± 1 µm (6 h) (Fig. 5e) & (Fig. 6e). The amplitude images clearly depict perceptive structural changes like formation of groove and perturbations at 1 h (Fig. 5-b, d) of exposure to water containing 0.077 mM of copper ions in the aqueous system.

Further exposure for an hour (i.e. total 2 h of exposure time) resulted in increased surface corrugation (number of grooves) (Fig. 5-b, d) and escalated average roughness from 5.04 ± 0.9 nm to 7.2 ± 0.8 nm (Fig. 7), with the rod- shape of the cell still being maintained (Fig. 5). At 3 h of exposure time the cell morphology altered significantly causing an elongation from 2.0 ± 0.5 (Control) to 3.0 ± 0.3 µm (Fig. 5e). The surface of the bacterial membrane was also significantly perturbed with the increase in average surface roughness to 9.4 ± 0.6 nm (Fig. 7) with a possibility of permeation (Fig. 5-b, d).

Further elongation in some cells, while collapse of cell edge with debris from the bacterial periplasm in few other cells; as evident from the phase image (Fig. 5c) was observed at 4 h of exposure time (Fig. 5). Vesicle formation advanced through time with an average roughness of 15.36 ± 0.9 nm ($p < 0.01$) (Fig. 7) and the complete cell collapse with leakage of cytoplasmic components from the apical ends (Fig. 6) as seen at 5 h and 6 h, respectively.

The phase images (Fig. 5c) & (Fig. 6c) for all the time line study enables us to visualize the variation in the stiffness across the cell (darker region indicating more compliant part of the cell). The finding of the present study offer significant information on the oligodynamic action of copper through conventional kill kinetics and topographical perturbations as a function of copper leaching time.

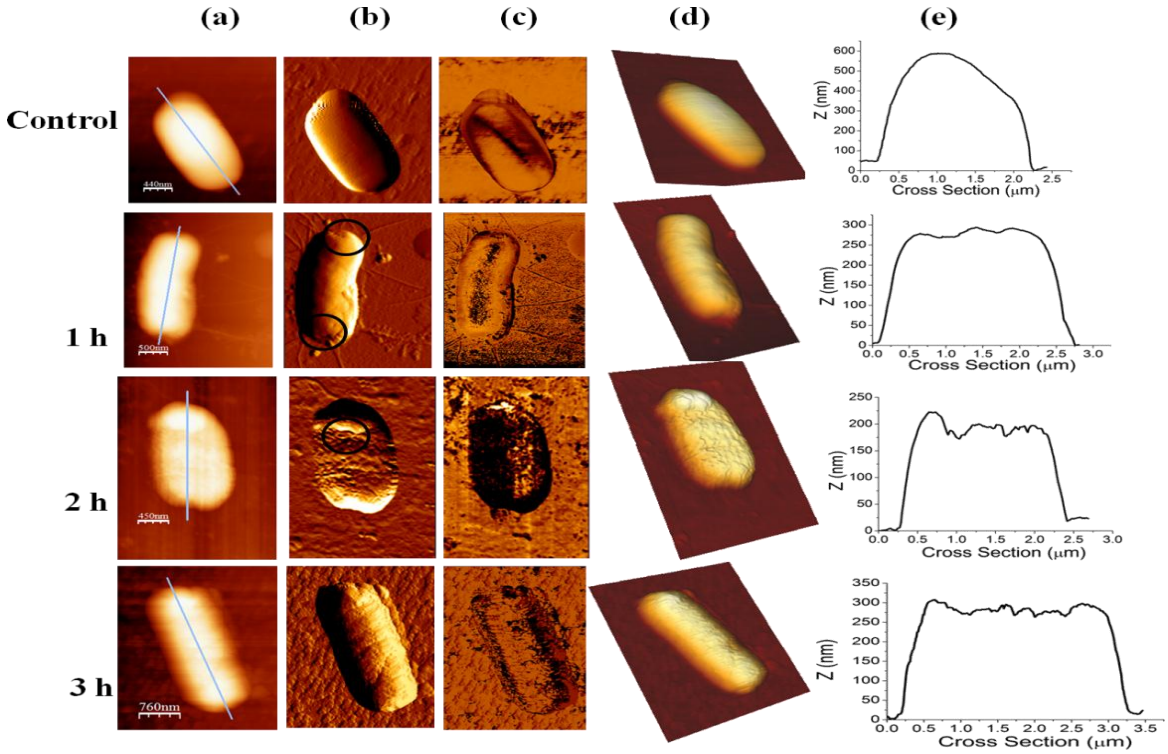


Fig. 5. AFM of *E. coli* untreated (Control) and exposed to copper ions leached in water at 1, 2 & 3 h: (a) topography, (b) amplitude, (c) phase, (d) three dimensional and (e) cross section images.

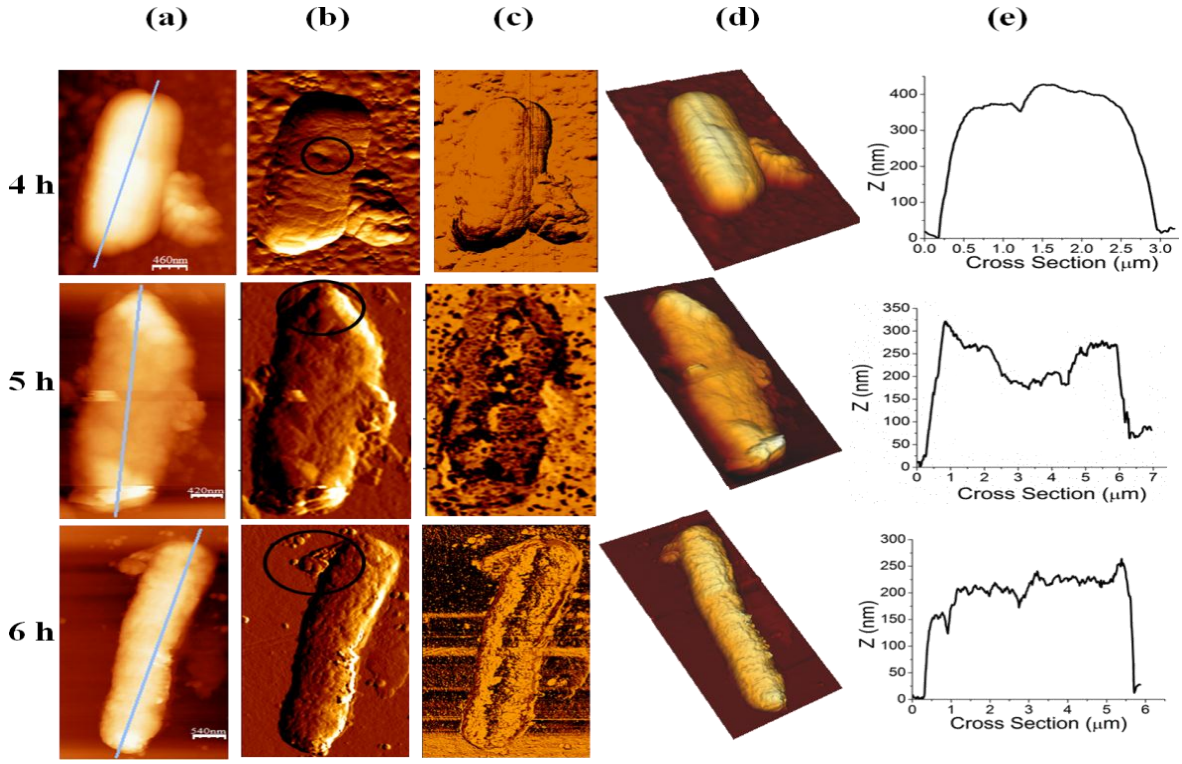


Fig. 6. AFM of *E. coli* exposed to copper ions leached in water at 4, 5 & 6 h: (a) topography, (b) amplitude, (c) phase, (d) three dimensional and (e) cross section images.

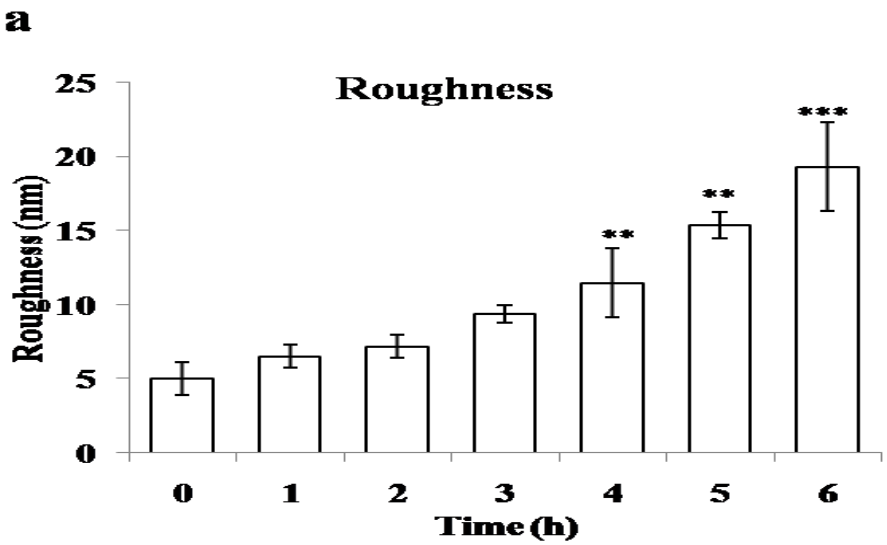


Fig. 7. The *E. coli* cell surface roughness as a function of leaching time on exposure to copper (- p < 0.01, ***- p < 0.005).**

4. DISCUSSION

Copper with its vast array of biological activities has been explored as one of the interesting elements for biomedical applications (Noycea et al., 2006; Mehtar et al., 2008; Szymański et al., 2012). Earlier, Mikihoro *et al.*, (2005) had proposed antimicrobial effect of metal ions exhibited by binding to DNA, enzymes and cellular proteins in bacteria, causing cell damage and death. Later studies on copper surfaces indicated that the hydroxyl radicals in the solution were responsible for their lethal action (Espirito Santo et al., 2008). The oligodynamic action of copper and silver has been known and studied with a precise correlation of the metal ions being responsible for the inactivation of the bacteria; however there is no agreement on its clear mechanism (Davies et al., 1997; Feng et al., 2000; Tandon et al., 2005; Shrestha et al., 2009; Landsdown, 2010; Varkey 2010; Li et al., 2013). Also, Liu et al., (2004) reported the effect of rare earth metals such as La^{3+} on *E. coli* damaging the cell's outer membrane due to the replacement of Ca^{2+} with La^{3+}. Also, so far no attempts have been made to visualize and correlate bacterial cell stress culminating in cell death as a result of copper ions in water with leaching time. It is evident from the present study that the treatment of *E. coli* with copper ions leads to dramatic morphostructural changes in the cell. Earlier investigators reported cell morphology after treating cells either with salts of copper, copper nanoparticles or copper-contact-surface using microscopic techniques other than AFM, such as confocal, SEM and TEM (Du et al., 2008; Raffi et al., 2010; Chatterjee et al., 2012). Although a few reports are available on the use of AFM to study the effect of copper on bacteria (Nan et al., 2008), none

of these have addressed a time line effect of ionic copper in correlation to its gradual internalization as in the present study.

Application of AFM has been reported in understanding the morphostructural effects as well as surface alterations caused by the influence of various antibiotics, AMPs, oils, nanoparticles and metal ions (Kasas et al., 1994; Braga and Ricci, 1998; Yang et al., 2006; Alsteen et al., 2008; Francius et al., 2008; Farmosa et al., 2012; Braga and Ricci, 2002; Eaton et al., 2008; Lui et al., 2010). Yang et al., 2010 demonstrated the effect of MIC of silver ions (bulk concentration) on cell communities of *E. coli* and *S. epidermidis* incubated for different time. The AFM topography and phase image of *E. coli* showed appearance of few vesicles on the cell surface on 1 h incubation followed by increased number of vesicles after 2 h. Further incubation of 3 and 4 h showed large vesicles and the loss of rod shaped cells morphology, respectively. On the contrary, in the present study *E. coli* cells were exposed to gradually built-up of copper ions in the system revealing a diverse action at 1 h showing groove formation but no vesicle formations on the cell surface as observed on gram positive *S. epidermidis* treated with silver ions (Yang et al., 2010). Similar effect of groove formation with corrugated surfaces were reported by Yang et al., (2006) on gram negative *E. coli* in response to the exposure to an antibiotic oflaxacin as well as with low concentration of sushi peptides (Li et al., 2007) and SWCNTs (Liu et al., 2010). Increased surface corrugation along with cell elongation was evident on further treatment of copper ions for 3 h indicating initiation of stress in the organism, alike the morphological changes induced by the treatment of cefodizime on *E. coli* cells (Braga and Ricci, 1998). This was also evident from the cell viability assay using confocal and standard microbiological procedures performed in the present investigation. Our study also revealed that *E. coli* treated with copper ions on incubation of 4 h show further increase in the cell surface corrugation with collapsed membrane edges as seen in the phase image which is similar to the amoxicillin-treated *E. coli* (Deupree et al., 2009). The AFM images of *E. coli* exposed to copper ions for 5 and 6 h, exhibit flattened cells, with extremely rough surfaces indicating complete collapse with leakage of intracellular contents from the apical ends of the filamented cells, leaving small amounts of cytoplasm. A similar effect was observed by treatment of organisms to supra-MIC concentration of cefodizime (Braga and Ricci, 2002), cationic AMPS magainin 2, melittin, PGLa, sushi 3 peptides (Meincken et al., 2005; Da Silva et al., 2003; Li et al., 2007). It could be postulated that the copper ions in the aqueous medium bind to the negatively charged LPS of gram negative bacterium penetrating into the bacterial cell. Since it has been

reported that copper ions posses the ability to chelate biomolecules or replace metal ions in specific metalloproteins, and thus disrupting metal cation homeostasis within the cells, resulting in bacterial toxicity (Chang et al., 2012). This leads to accumulation of stress within the bacterial outer membrane, attributing to the leakage of the cytoplasmic material from the apical ends. Our study thus stands apart by visually demonstrating the probable anti-bacterial mechanism exhibited by gradually internalised copper ions leached in aqueous medium and understanding the relationship between morphology and function of microbial cells.

5. CONCLUSIONS

In summary, our results using AAS show that copper concentration inside *E. coli* cells increased with Cu ion exposure time. The AFM and confocal microscopy results show the appearance of groves on the cell surface, cell elongation and ultimately the loss of membrane integrity. To the best of our knowledge we have given a direct evidence of an inside-out death pathway with copper ion internalization in *E. coli*. AFM images clearly indicated the perceptible stress built-up which corroborated our microbiological assay and confocal microscopy findings, successfully emphasizing the use of copper as an effective alternative to the existing antimicrobial agents.

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