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REGULAR ARTICLE

EVALUATION OF ANTIBACTERIAL ACTIVITY OF ZnO NANOPARTICLES COATED SONOCHEMICALLY ONTO TEXTILE FABRICS

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ABSTRACT

Growing resistance of microorganisms to potent antibiotics has renewed a great interest towards investigating bactericidal properties of nanoparticles and their nano-composites as an alternative. In the present work studies have been carried out to investigate the antibacterial properties of ZnO nanoparticles (NPs). Various tests were performed to assess the antibacterial activity of cotton fabrics coated with ZnO nanoparticles against Gram positive *Staphylococcus aureus* and Gram negative *Escherichia coli*. The antibacterial activities of the fabrics were assessed semi-quantitatively by the agar diffusion method and the shake flask method (nutrient broth) and quantitatively by the shake flask method (saline) and the absorption method (ISO 20743:2007). The results showed a significant antibacterial activity of ZnO nanoparticles coated onto fabrics against both bacteria, with a slightly higher activity against *Staphylococcus aureus* as compared to *Escherichia coli*.

Keywords: Antibacterial textiles; zinc oxide nanoparticles; antimicrobial finish

INTRODUCTION

Nosocomial infections, or hospital acquired infections, are associated with treatment in a hospital or a health care setting. An infection is considered to be nosocomial if it appears after 48 hours of hospital admission or within 30 days of discharge. Improved hygiene and infection control programs have proved to be very successful in reducing rates of nosocomial infections. A wide range of bacteria, fungi and viral pathogens are responsible for such infections **(Horan *et al*., 1993)**. Many of these organisms are also part of the commensal flora which coexists in a natural equilibrium with the human body. A rapid and uncontrolled multiplication of these pathogenic microbes can seriously compromise health and hygienic living standards. One potential method for reducing the occurrence and spread of nosocomial infections is the use of antimicrobial textiles. In terms of transmission, textiles play a crucial role in the chain of infection for pathogenic microorganisms. Textiles of all varieties are considered to be very proficient at carrying bacteria and serving as a reservoir for the transmission of infection **(Tinker, 2010)**. Therefore, antimicrobial textiles may be of great help in the recovery process of transplant patients, people with immunodeficiency diseases, low immunity patients and premature babies **(Tinker, 2010)**. Antimicrobial garments may also be useful for individuals coming in to contact with patients, such as visitors, nurses, doctors and other healthcare workers.

The Sonochemistry centre at Coventry University is one of a group of 17 partner organisations working on a EU Framework 7 (FP 7) funded programme, aimed at developing a pilot line industrial process for the coating of antimicrobial nanoparticles on to textile surfaces by ultrasound. The technology under development is based on a lab scale sonochemical process that was developed at Bar-Ilan University (BIU), Israel. The technique uses a single step process to synthesize and impregnate antibacterial nanoparticles on to fabrics by the use of ultrasonic waves. “Ultrasonic irradiation has been proven to be an effective method of deposition of nanoparticles on the surface of various substrates including ceramics and polymers” **(Abramov *et al*., 2009)**. This process results in a smooth and homogeneous layer of coating and is capable of projecting nanoparticles towards the fabric surface at a very high speed. The high impact speed causes them to adhere strongly to surfaces **(Abramov *et al*., 2009)**. It is hoped that these textiles can be produced at an affordable price for routine use in hospitals as bandages, hospital sheets and uniforms. The simultaneous synthesis and coating of antimicrobial fabrics reduces the number of production steps and thus should reduce the energy and costs required for production. It should also be

possible to recycle the majority of the chemicals for reuse in the process rather than disposal. This kind of processing is quite practical and durable, as the antimicrobial agent is physically incorporated in the textile structure and only released slowly during use.

Antimicrobial agents are natural or synthetic compounds that inhibit microbial growth. Various classes of antimicrobial agents are used in the textile industry, most of which are biocides. The use of inorganic nanoparticles has advanced rapidly due to the amount of work done towards the synthesis and modification of particles for biomedical applications. Many heavy metals and metal oxides either in their free state, or in compounds at very low concentrations, are toxic to microbes **(Padmavathy and Vijayaraghavan, 2008)**. These inorganic materials kill bacteria through various mechanisms, such as by binding to intracellular proteins and inactivating them, generation of reactive oxygen species and via direct damage to cell walls (Gao and Cranston, 2008). Zinc oxide (ZnO), copper oxide (CuO), magnesium oxide (MgO), titanium dioxide (TiO_2) and silver (Ag) are some of the most commonly used inorganic materials in the fabrication of antimicrobial coatings.

Nanoparticles are particles that have one dimension that is 100 nanometers or less in size. The formation of nanoparticles changes the properties of many conventional materials **(Reddy *et al*., 2007).** For example, the larger surface area of nanoparticles can result in a greater degree of interaction with bacterial cell walls. Amongst inorganic materials, metals and metal oxides have elicited a great deal of interest for use as antimicrobial agents because of their durability, selectivity and resistance to heat. One of the reasons why these inorganic materials (metals and metal oxides) have attracted so much attention is their ability to withstand intensive processing conditions **(Fu *et al*., 2005; Makhluf *et al*., 2005)**. They have selective toxicity to bacteria but have minimal effects on human cells **(Reddy *et al*., 2007)**.

Zinc oxide (ZnO) belongs to a group of metal oxides with photo-oxidising and photocatalytic ability against chemical and biological species **(Szabó *et al*., 2003)**. It is an inorganic white powder and is insoluble in water. ZnO nanoparticles have been shown to be useful antibacterial and antifungal agents when used as a surface coating on materials and textiles **(Abramov *et al*., 2009)**. Zinc is an essential element and ZnO nanoparticles are considered to be non-toxic. Toxicity studies have shown that zinc ions do not cause any damage to the DNA of human cells **(Yamada *et al*., 2007)**. A study by Yamamoto **(2001)**, to evaluate the antibacterial activity of ZnO with different particle sizes showed that ZnO nanoparticles (10-50 nm) exhibit better antimicrobial properties than bulk ZnO (2 μm). The antibacterial activity of ZnO nanoparticles is due in part to their electrostatic interaction with cell surfaces. Sharma *et al*. **(2010)**, showed that on contact with bacteria, the cytotoxic

behaviour of ZnO nanoparticles ruptures the lipid bilayer of bacterium resulting in leakage of cytoplasmic contents.

The present study was carried out with an objective to characterize the antimicrobial properties of cotton fabrics finished with ZnO nanoparticles against a variety of bacterial strains commonly associated with nosocomial infections.

MATERIAL AND METHODS

Materials

Reagents used in all tests were of analytical quality. Oxoid dehydrated agar and broth powders were used to prepare culture media and the Oxoid manufacturer's instructions were followed for media preparations. Water was of analytical grade; deionised water was used to prepare all microbiological media. Soybean–Casein Digest broth with Lecithin and Polysorbate 80 (SCDLP) medium was prepared by dissolving 17g of casein peptone, 3g of soybean peptone, 5g of sodium chloride, 2.5 g of potassium dihydrogen phosphate, 2.5 g of glucose, 1g of lecithin and 7g polysorbate 80 (non-ionic surfactant) in 1 litre of distilled water. All contents were mixed thoroughly on a shaker. After mixing, it was sterilized by autoclaving at 121°C for 15 minutes. Prior to autoclaving, the pH was adjusted to around 7.2 at room temperature using sodium hydroxide and sterilized again by autoclaving 121°C for 15 minutes.

Preparation of Fabrics

The ZnO coated cotton fabric was prepared using a laboratory coating system at Bar-Ilan University (BIU) in Israel in accordance to their previously published procedure **(Abramov *et al*., 2009)**. Zinc acetate powder was dissolved in a 9:1 mixture of ethanol and water and placed in the coating tank. The ultrasonic transducers were turned on in order to heat the solution to 55 ± 5°C. Then ammonium hydroxide was added drop wise until the solution reached a pH of 8. Rolls of cotton (10 m x 0.1 m, plain unbleached woven cotton 145 $g.cm^{-2}$) were then fed at a constant speed (22 $cm.min^{-1}$) through the coating tank and ultrasonic field. After coating, the rolls of cotton were washed with clean water and ethanol, and then dried **(Abramov *et al*., 2009).**

Test microorganisms

The bacterial species used here included Gram negative *Escherichia coli* (ATCC 8739) and Gram positive *Staphylococcus aureus* (ATCC 6538). Bacteria were maintained on nutrient agar at 37^{o}C.

Preparation and incubation of test inoculums

The bacterial suspensions were prepared by taking a single colony from the stock bacterial culture with a loop and inoculating 20 ml of sterile nutrient broth in a 100 ml Erlenmeyer flask. The flask was then incubated in a shaking incubator at 37°C at 110 rpm for 24 ± 2 hours.

After incubation, 0.4 ml of the overnight inoculum was transferred to a 100 ml Erlenmeyer flask containing 20 ml nutrient broth and incubated in a shaking incubator for 3 hours at 37°C and 110 rpm. One ml was then serially diluted 3 fold in nutrient broth to obtain an approximate target concentration of between 1 x 10^5 colony forming units/ ml (CFU.ml^{-1}) and 3 x 10^5 CFU.ml^{-1}. The number of bacteria in the 3 hour culture was estimated by measuring the optical density of the culture at 660 nm (Corning colorimeter 253). An optical density of between 0.1 and 0.3 was roughly equal to a concentration of between 1 x 10^8 CFU.ml^{-1} and 3 x 10^8 CFU.ml^{-1}.

Agar diffusion method (Semi-quantitative test)

The agar diffusion method (Kirby-Bauer) is a relatively quick and easily executed semi-quantitative test to determine antibacterial activity of diffusible antimicrobial agents on treated textile material. The bacteria were grown in nutrient broth medium. Using x100 dilution from a 3 hour culture, test organisms were swabbed over the surface of Isosensitest agar plates (Oxoid). 10 mm diameter discs of the test fabric and control fabric were then gently pressed on to the surface of the plate. The plates were incubated at 37°C for 18 - 24 hours. The antibacterial activity of fabrics was demonstrated by the diameter of the zone of inhibition in comparison to the control fabric. Images were recorded using a Protocol 2 plate counter (Synbiosis, UK.). The experiment was performed in a duplicate and the mean value was taken.

Shake flask test in nutrient broth (Semi-quantitative test)

The antibacterial activity was also evaluated using a shake flask method with nutrient broth. This method was used to monitor growth inhibition by the fabrics. The samples were placed in shaking flasks for 3 hours at 37°C and the change in optical absorbance over time was monitored. Test and control fabrics were cut into small pieces of 5 x 5 cm (0.4 g) and placed into empty sterile Erlenmeyer flasks in triplicates. The fabrics were inoculated with 0.2 ml of bacterial inoculums (x100 dilution of a 3 hour culture). Immediately after inoculation, the fabrics were soaked in 20 ml of nutrient broth. The flasks were then shaken at 110 rpm in a rotary shaking incubator at 37°C. The absorbance was checked at 660 nm from time 0 to 3 hours with intervals of 30 minutes. A final reading was also taken at 24 hours following overnight incubation. Antimicrobial efficacy was determined based on triplicate test results.

Shake flask test in saline (Quantitative test)

A shake flask method was also performed in saline to investigate the rate of reduction in the number of bacteria. A different medium (saline) was used to evaluate the growth of microbes without an added nutrient source. Fabric samples were placed in contact with 20 ml of sterile saline (0.85% NaCl) in Erlenmeyer flasks in triplicates. Fabrics were inoculated with 1 ml of bacterial inoculums. The inoculums were x100 dilution in saline of a 3 hour culture. The tests against each bacterium included (a) control fabric in saline (b) test fabric in saline (c) no fabric in saline. All the three treatments were performed in triplicate. The flasks were then subjected to incubation in a rotary shaking incubator at 37 ± 2°C. The number of viable cells after incubation was obtained by spread plating serial dilutions on nutrient agar at 0, 1, 3 and 24 hours.

Absorption method (ISO 20743:2007)

This method of evaluation involves testing bacterial suspension directly inoculated onto textile samples. The test was performed in accordance to ISO 20743:2007 standards **(ISO, 2010)**. Bacterial suspensions were prepared by taking a single colony from the stock bacterial culture with a loop and inoculating 20ml of sterile nutrient broth in a 100ml Erlenmeyer flask. The flask was then incubated in a shaking incubator at 37°C at 110rpm for 24 hours. After incubation, 0.4ml of the overnight inoculum was transferred to a 100ml

Erlenmeyer flask containing 20ml nutrient broth and incubated in a shaking incubator for 3 hours at 37°C and 110rpm to obtain an expected target CFU concentration of 10^8 CFU.ml^{-1}. The optical absorbance of the culture at 660 nm was checked (0.1 – 0.3) and it was serially diluted 3 fold in dilute nutrient broth (1 in 20 dilution in water). The resulting dilution contained a concentration of bacteria of between 1 x 10^5 and 3 x 10^5 CFU.ml^{-1}.

A 0.2 ml aliquot of bacterial inoculum was pipetted over the surface of each control and each test fabric drop. Care was taken not to touch the surface of the vial. Immediately after inoculating the samples, 3 control and 3 test fabrics were mixed with 20 ml of neutralizing medium (SCDLP). The vials were tightly capped and shaken vigorously by hand in an arc of ~30 cm for 30 seconds. The remaining universals with the 3 control and 3 test fabrics were incubated at 37°C for 18 - 24 hours immediately after inoculation with the test bacterium. After incubation the control and test fabrics were treated in the same way with SCDLP.

A 3 fold dilution series was prepared for the initial set of control and test fabrics. Petri dishes containing agar were pre-labelled (10^0, 10^{-1}, 10^{-2} and 10^{-3}) for the initial set of control and test fabrics and a 5 fold dilution series was prepared for the post incubation set.

One ml was taken from each dilution tube and pipetted onto two corresponding Petri dishes in a zig zag manner across the plate. After pipetting 1ml of inoculum from all serially diluted broth tubes, approximately 17 ml of molten PCA (45 ± 3°C water bath) was added to each Petri dish. The Petri dishes were rotated gently to ensure that the culture and medium was thoroughly mixed, and that the medium evenly covered the plate. Plates were left at room temperature until the medium solidified, then turned upside down to ensure water condensation did not fall on to the agar. The plates were then incubated at 37°C ± 2°C for 18 – 24 hours. The numbers of colonies were counted to calculate bacterial growth values for the control and test fabrics. The antibacterial efficiency value (A) was calculated using the following formula:

$$\mathbf{A = F - G}$$

Where, **F** - growth value on the control fabric sample (log CFU.ml^{-1} post incubation - log CFU.ml^{-1} prior to incubation) and **G** - growth value on the treated fabric samples.

RESULTS AND DISCUSSION

The antibacterial activity of ZnO nanoparticles was assessed using 4 different methods in order to investigate the efficacy under different conditions. The ability of the antibacterial

agent to inhibit bacterial growth was first tested using a disc diffusion method. Cotton discs (10 mm) with or without the ZnO nanoparticle coating, were tested and the results are shown in Table 1. Figure 1 shows a clear zone of inhibition around ZnO treated fabric disc where as the control fabric disc shows no sign of inhibition. The greatest inhibitory effect was observed against *Staphylococcus aureus* with a zone of inhibition of 18.5 mm diameter followed by *Escherichia coli* with a zone of inhibition of 13.5 mm diameter.

Table 1 Antibaterial assessment by disc diffusion method

Fabrics Treated	Organism	Zone of Inhibition (mm) (Diameter)			
		1	2	Mean	St. Dev
ZnO	***S. aureus***	18	19	18.5	0.71
	E. coli	13	14	13.5	0.71
Control	***S. aureus***	0	0	0	0
	E. coli	0	0	0	0

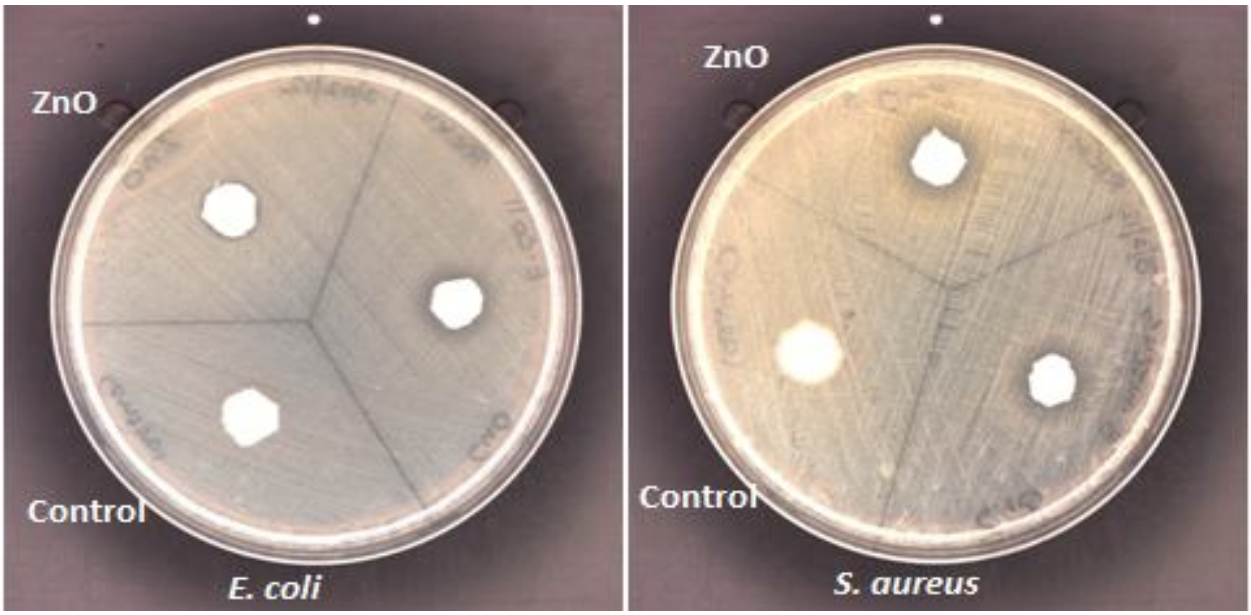

Figure 1 Antibacterial activity of ZnO nanoparticle treated fabrics showing zone of inhibition against tested bacteria by a disc diffusion method

The antibacterial efficacy of the ZnO coated cotton was also determined using two shake flask methods; one with nutrient broth as the medium, and one with saline as the medium. In the case of the nutrient broth test, bacterial growth was monitored by measuring the optical density of the medium over time. The percentage microbial reduction with the ZnO coated fabrics against both the species of bacteria is shown in Table 2.

In Figure 2 graphs are included showing the change in absorbance over time. A very high antimicrobial activity was seen against both bacteria. Absorbance measurements are not as accurate as plate counts for the determination of viable bacteria but they can give a rapid estimate of cell numbers. Absorbance measurements (turbidity) are commonly used for minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) tests.

Using the absorbance value shown in Table 2 there was a 97% reduction in growth for *Staphylococcus aureus* with the ZnO followed by an 87% reduction in growth for *Escherichia coli*. The dynamic shake flask method was developed for routine quality control and screening tests in order to overcome difficulties in using conventional antimicrobial test methods such as ensuring proper contact of inoculums to treated surface **(ASTM, 2010)**. This variant of the shake flask method is quicker to perform due to the use of absorbance measurements rather than conventional plating method. It gives an indication of bacterial efficiency within hours where as it takes at least 3 days to get the results by conventional plating methods.

Table 2 Results of nutrient broth shake flask test in terms of percentage growth reduction

Test Organism	O.D. after 3 hours (660 nm)		% Reduction	O.D after 24 hours (660 nm)		% Reduction
	Control	ZnO coated		Control	ZnO coated	
S. aureus	0.0218	0.0006	97.24	1.665	0.002	99.87
E. coli	0.0770	0.0103	86.62	2.1137	0.0037	99.82

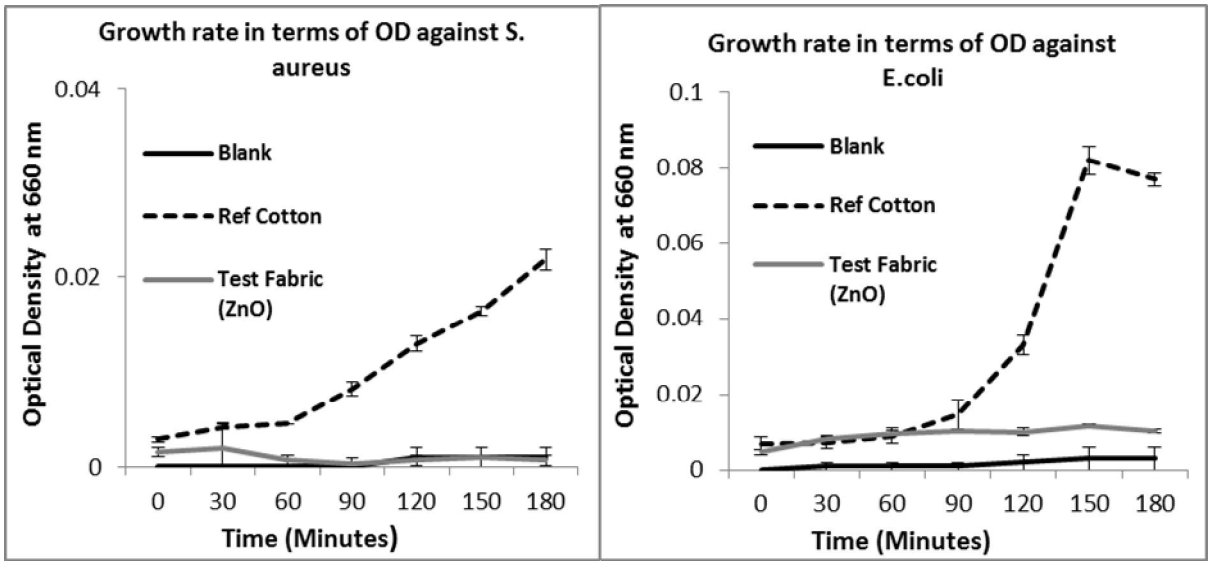

Figure 2 Graphs showing change in absorbance over time (3 hours) for nutrient broth shake flask tests. Values poltted are mean ± SD, n = (3)

A shake flask method was also performed with saline rather than nutrient broth. This method is closer to the standard dynamic shake flask method **(ASTM, 2010)** in that no nutrient source is added. In this method bacterial growth is much slower and so absorbance measurements are not suitable for monitoring bacterial numbers. Instead standard plate counts are used. The results from these tests are shown in Figure 3. A strong bacteriocidal activity was observed against both *Staphylococcus aureus* and *Escherichia coli*. However, higher activity was seen against *E. coli* than *Staphylococcus aureus*. For *Escherichia coli*, ZnO nanoparticles reduced bacterial numbers to undetectable levels within one hour of contact where as there were still viable *Staphylococcus aureus* after 1 hours incubation. In the case of *Staphylococcus aureus,* there was also some inhibition of growth with the control cotton. In other tests (ISO method below) this inhibition has been found to be removed by rinsing the cotton prior to use. The actual cause of the inhibition has not been identified but was specific to *Staphylococcus aureus.*

Both of the shake flask methods have advantages and disadvantages. The saline method is less susceptible to contamination than the nutrient broth method because of the lack of nutrients and consequently slower growth. It is however more time consuming due to the need for standard agar plating to enumerate bacterial numbers. The nutrient broth method is similar to MIC and MBC test methods in the use of a nutrient medium, growth inhibition is assessed under conditions that are highly favourable to growth (nutrients available). The saline method more closely resembles actual working conditions where in most cases

nutrients will be in short supply. The last method used here, the absorption method from ISO 20743 **(ISO, 2010)**, is similar to the saline shake flask test in that nutrients are not added to support the growth of the bacteria. It is intended to better simulate actual working conditions for antibacterial fabrics. A sample of bacterial inoculum is allowed to absorb in to the fabric, then the fabric and bacteria are incubated together and finally post incubation, any viable cells are recovered and enumerated.

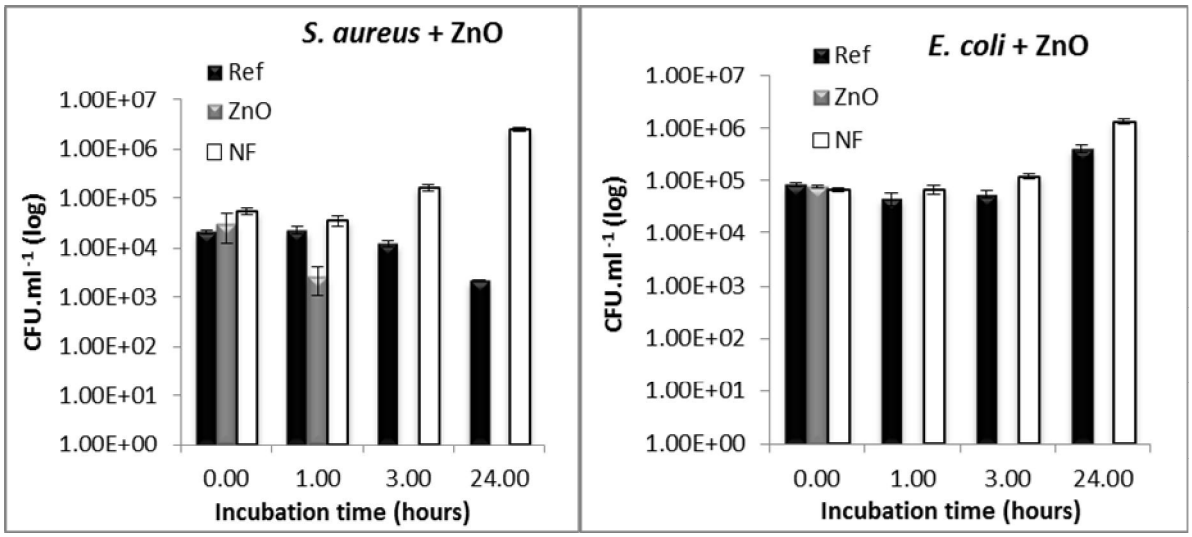

Figure 3 Graphs showing antibacterial efficiency of ZnO coated fabrics against different bacteria using a saline shake flask test method. Values shown are mean ±SD, n = (3)

For this analysis, 6 ZnO coated test pieces and 6 control pieces (all 5 x 5 cm) were challenged with *Escherichia coli* and *Staphylococcus aureus* and tested through the ISO 20743 absorption method **(ISO, 2010)**. The viable bacterial counts recovered from the fabrics after 24 hours of contact time are shown in Table 3. As shown in Figure 4, the control samples supported bacterial growth and as expected did not cause any bacterial killing during the 24 hour contact time. In the case of the cotton with ZnO nanoparticles a significant bacteriostatic effect was observed. There was a greater than 99% reduction in the growth of both *Staphylococcus aureus* and *Escherichia coli* during the 24 hour incubation. These results agree with the results from the agar diffusion tests and the shake flask test methods in terms of a positive antibacterial effect. However, the saline shake flask results indicated a full bacterial kill (bactericidal effect) rather than simply growth inhibition (bacteriostatic effect). In the saline shake flask tests, a full bacterial kill was observed within 3 hours of contact time, but in the absorption tests, viable bacteria were recovered from the test samples after the 24 hour incubation. One possible reason for this is that there was not direct contact between some of

the inoculum and the surface of the fabric. Some of the incoulum may have wet the sides of the Universal tube rather than the fabric allowing some of the bacteria to survive the incubation phase.

A study by Yamamoto *et al*. **(2000)**, stated that the presence of reactive oxygen species (ROS) generated by ZnO nanoparticles is responsible for their bactericidal activity. Zhang *et al*. **(2010)**, further showed that the antibacterial behaviour of ZnO nanoparticles could be due to chemical interactions between hydrogen peroxide and membrane proteins, or between other chemical species produced in the presence of ZnO nanoparticles and the outer lipid bilayer of bacteria. The hydrogen peroxide produced enters the cell membrane of bacteria and kills them. It was shown in the study that nano sized ZnO particles are responsible for inhibiting bacterial growth **(Zhang *et al*., 2010)**. Padmavathy and Vijayaraghavan **(2008)**, further elucidated the bactericidal activity of ZnO nanoparticles. According to them, once hydrogen peroxide is generated by ZnO nanoparticles, the nanoparticles remains in contact with the dead bacteria to prevent further bacterial action and continue to generate and discharge hydrogen peroxide to the medim **(Padmavathy and Vijayaraghavan, 2008)**. Our results correspond with the results of the authors above, showing that ZnO nanoparticles applied on to fabrics have an excellent antimicrobial activity.

Table 3 Viable bacterial counts (CFU/ml) after 24 hours contact time showing log reduction in treated sample compared to untreated sample. CFU/ml at time zero 1 – 3 x105 CFU/ml (*Nanoparticles)

Bacteria	**After 24 h contact time**		**Log reduction**	**% Reduction**
	Untreated	**ZnO (*) treated**		
S. aureus	5.40E+07	1.20E+04	3 log reduction	99.98
E. coli	8.00E+08	9.30E+05	3 log reduction	99.88

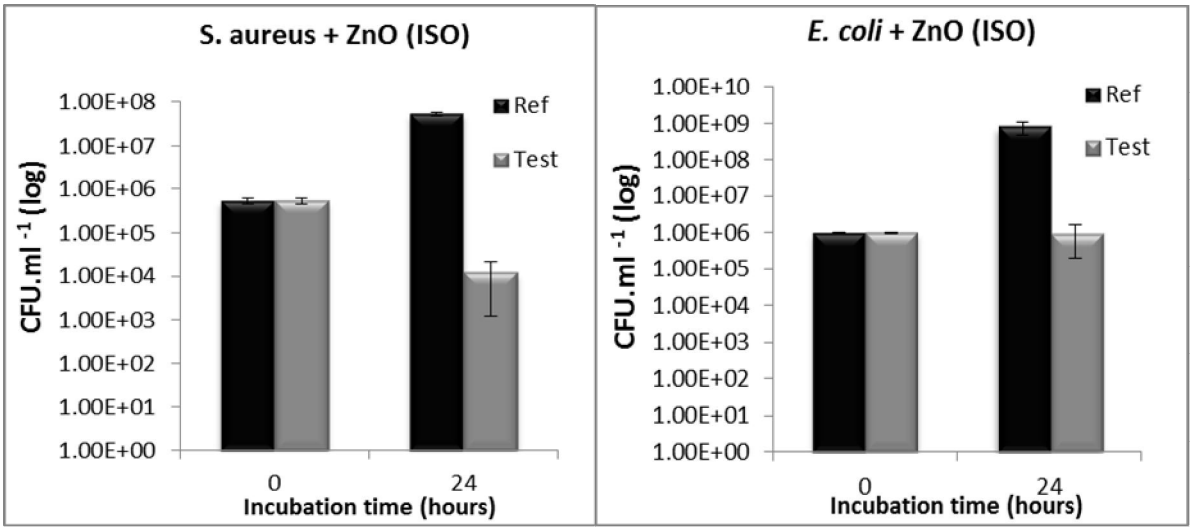

Figure 4 Results of antimicrobial assessments by the absorption method from ISO (20743)

CONCLUSION

The cotton fabrics sonochemically coated with ZnO nanoparticles showed a very good degree of antibacterial activity when compared to control untreated cotton fabric. Overall the results demonstrated a slightly higher antibacterial activity against *Staphylococcus aureus* than *Escherichia coli*.

The results from this work are aiding the scale-up of a sonochemical coating process for the production of efficient and economical antibacterial fabrics. These fabrics will be a useful tool in hospitals as an additional weapon in the fight against hospital acquired infections.

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