

Effectivity of Titanium Oxide Based Nano Particles on *E. coli* from Clinical Samples

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ABSTRACT

Introduction: Nanoparticles composed of Titanium Oxide (TiO₂) are non toxic, durable, stable and have a high refractive index with a lot of scope in biomedical applications. Due to their antibacterial effects, they can be applied to inanimate objects like glass, metal and even biomedical implants.

Aim: This study was conducted to assess the antibacterial effect of Titanium Oxide (TiO₂) alone or with Silver (Ag) as an additive on *Escherichia coli*.

Materials and Methods: *Escherichia coli* isolates (n=25) sensitive to most of the drugs including first generation cephalosporins, ampicillin and amoxycillin from various samples like pus, urine, sputum and blood were placed onto the glass slides containing TiO₂ annealed at 200°C, 400°C, TiO₂ with 0.1% Ag as additive, TiO₂ with 0.3% Ag, and TiO₂ with 0.6% Ag as additive. Samples from this were inoculated at every hour onto sterile petri plates

and observed for growth after overnight incubation at 37°C.

Results: The organisms which were inoculated onto TiO₂ annealed at 200°C showed a slower reduction rate from >1 × 10⁸ cfu/ml to <1 × 10⁴ cfu/ml only after six hours of incubation in visible light. Complete absence of colony forming units was observed after eight hours of incubation. The samples treated with TiO₂ at 400°C showed no growth after six hours of incubation itself. Samples treated with TiO₂ with increasing gradations of silver as additives showed proportional reduction in the incubation time for the complete absence of colony forming units.

Conclusion: Our study shows that pure titanium oxide has a high antibacterial effect on pathogenic samples of *Escherichia coli* from clinical isolates, which is further increased with the addition of increasing concentrations of silver.

Keywords: Antibacterial effect, Biocidal, Metal oxide, Oxidative damage, Silver

INTRODUCTION

Surgical site infections are the second most common infections among hospital acquired infections causing high morbidity and mortality [1]. They account for a quarter of all nosocomial infections [2]. The infection can be acquired through healthcare workers or any other iatrogenic sources, including scalpels, forceps or body implants during surgeries or any other invasive procedures.

A high incidence rate of infection is observed in the use of implanted biomedical devices, such as bone fracture fixation pins, catheters and heart valves etc., which result in a tremendous increase in human health and healthcare costs [3]. Moreover, all these devices are highly exposed to infection by multiresistant bacteria, on which systemic antibiotics have very little effect [4,5]. For example, *Escherichia coli* is an especially difficult organism to treat as it can adapt to changing environmental conditions becoming resistant to variety of antibiotics and hence not eradicated completely even after treatment [6].

Hence, an alternative strategy is of utmost priority in order to control infections. Since, bacterial adhesion to biomaterial surfaces is an essential step in the process of infection, modifications to biomedical surfaces are considered to diminish infections by inhibiting initial bacterial adhesion [4,7].

Of late there is a lot of interest in the field of Nanomedicine wherein different types of nanoparticles are used for their antibacterial effect. Nanoparticles are objects of a minute size, measuring about 1-100 nanometers, but have the capacity to behave as a complete unit with respect to its transport and properties. They are further classified according to their shape and size into ultrafine, fine and coarse [8]. Interfacing the nanoparticles with biological molecules and structures may result in a major development in the treatment of various disorders.

In the recent years, nanoparticles, especially those composed of

TiO₂, is the object of considerable research due to their potential biomedical applications. They are known to be non toxic and highly durable with a high refractive index, apart from having antibacterial properties. Since TiO₂ are very stable, it is very easy to apply them to inanimate objects such as glass, metal and biomedical implants.

Different modes of action of the nanoparticles on bacteria have been described. One of them is said to be by oxidative damage on the cell membrane of the organism [9]. Another mode of action described is an alteration of the Coenzyme A dependent enzymes and their activity [10], while damage to the DNA by means of hydroxyl radicals have also been reported to be one of the causes [11].

It is well known that silver ions show good antibacterial properties without any toxic effects in comparison to other heavy metal ions. The biocidal activity of silver is related to the biologically active silver ion released from silver coatings. The silver ions (Ag⁺), have the capacity to bind to the proteins and enzymes present on the bacterial cell wall and the cell membrane. This leads to the disruption of the cell membrane which causes permeability imbalance, cellular disintegration, ultimately cell death [12,13]. It is also reported that silver has long played a role in the treatment of burns [14]. It has also been noted that the infection rate was effective not only in the humans but also in the animals when treated with silver-coated medicines [15].

Much of the work is done by the researchers on the self cleaning property of TiO₂ with or without doping of metal ions in water and in air. Most of the bacterial samples used in these studies are samples of standard strains such as American Type Culture Collection (ATCC) and National Collection of Industrial Microorganisms (NCIM) strains. Nevertheless here has been little work done on samples collected from the clinical samples of the patients.

Hence, in this present study, we have attempted to use *Escherichia coli*, which is one of the major pathogens of the humans capable

of causing diseases from different clinical samples and analyze the antibacterial effect of pure TiO₂ and TiO₂ with various concentrations of Ag as an additive.

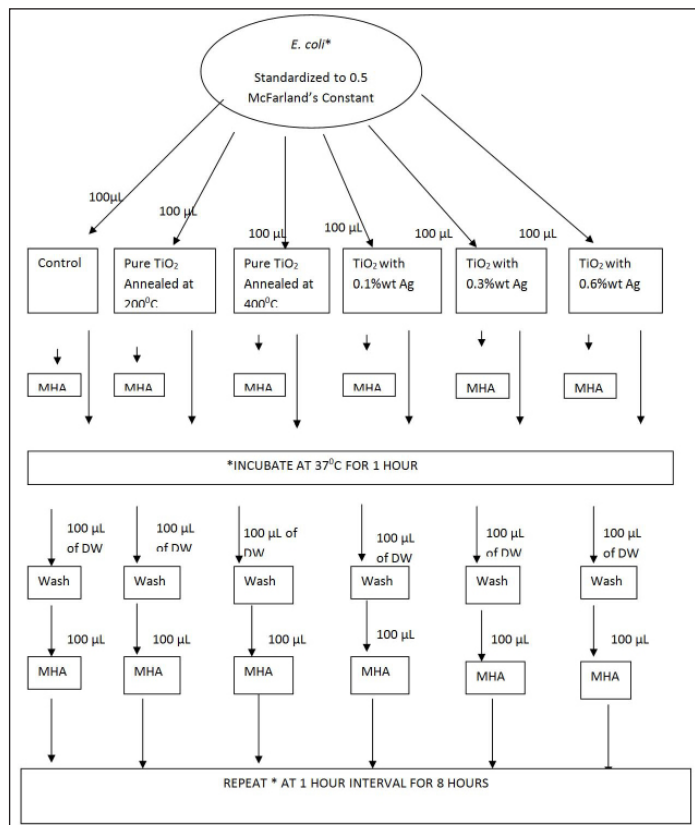
MATERIALS AND METHODS

This prospective study was performed at the Department of Microbiology of Malla Reddy Institute of Medical Sciences from September 2013 to July 2016.

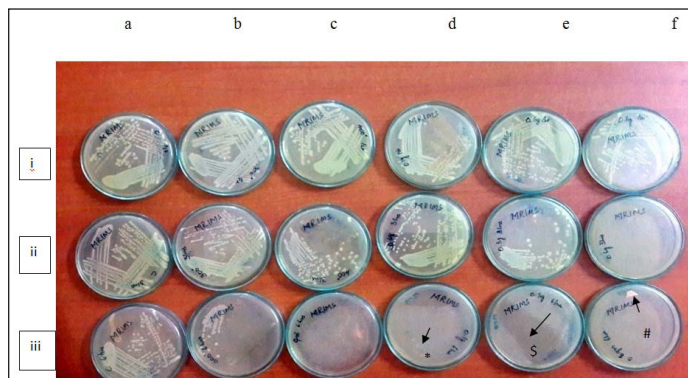
Inclusion criteria: Twenty five *E. coli* isolates sensitive to most of the drugs including first generation cephalosporins, ampicillin and amoxicillin from various samples like pus, urine, sputum and blood were included in the study.

Exclusion criteria: All other pathogenic bacteria which were isolated during the culture of the clinical isolates were excluded from the study.

Preparation of nanoprticles: Calculated quantity of titanium isopropoxide (Aldrich, 99.98% purity) was dissolved in 2-methoxy ethanol (99.98%) solvent along with acetyl-acetone as a complexing and chelating agent. 1.2 g of cetyltrimethylammonium bromide was dissolved in 20 ml of 2-methoxy ethanol solvent in beaker and the contents were added drop wise to the titanium isopropoxide sol under vigorous stirring. The contents were stirred at room temperature for about six hours. The molar ratio of titanium isopropoxide, 2-methoxy ethanol and acetyl-acetone were 2.0:10.0:0.5. To the above contents calculated quantities of HNO₃ (1.2 ml) and of deionizing water (1.5 ml) were added drop wise as a catalyst to increase the rate of reaction, and hydrolysis of the sol, respectively. Further the contents were refluxed at 70°C for three hours to complete the reaction and later cooled to room temperature. The contents were filtered using Whatman filter paper in order to remove any particulates formed during the reaction. The obtained stock solution is used for the deposition on to glass slides, quartz and silicon substrates by spin coating process [12]. Five sets of 25 glass slides precoated with nanoparticles by the above sol gel technique were taken into the study. Each set was pre coated with either pure TiO₂ annealed at 200°C, or at 400°C, or TiO₂ doped with 0.1%, 0.3%, 0.6% Silver (Ag).



[Table/Fig-1]: Flowchart of the inoculation procedure. *E. coli*- *Escherichia coli*, TiO₂- Titanium Oxide, Ag- Silver, MHA- Mueller Hinton Agar, DW – Distilled Water, µL – microliter

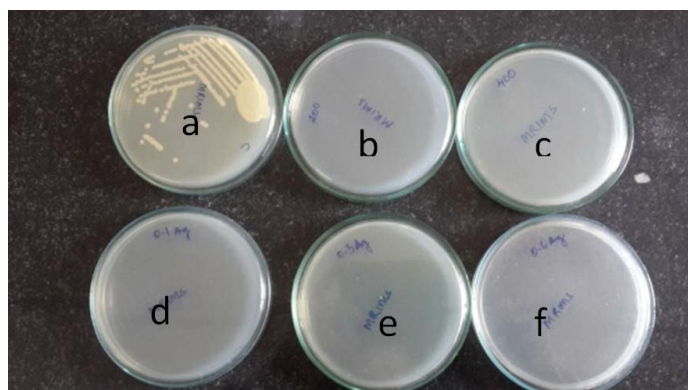


[Table/Fig-2]: Growth of *E. coli* treated with TiO₂ and TiO₂ with silver as additives. a: control, b: TiO₂ annealed at 200°C, c: TiO₂ annealed at 400°C, d: TiO₂ with 0.1% Ag, e: TiO₂ with 0.3% Ag, f: TiO₂ with 0.6% Ag
i. At 0 hours of incubation
ii. At 3 hours of incubation
iii. At 6 hours of incubation
* and #: slight contamination in the media, \$: shadow

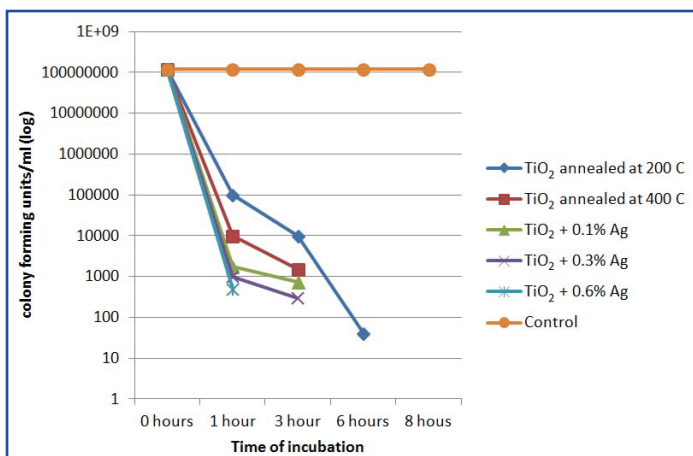
Antibacterial efficacy test: Each of the *E. coli* isolates was inoculated into peptone water and incubated at 37°C overnight. Next day, the turbidity was standardized to that of 0.5 McFarland standards. This was considered to be the working broth. Almost 100 µL of this broth was added onto one glass slide from each set, i.e., one coated with TiO₂ annealed at 200°C, one annealed at 400°C, one each with TiO₂ with 0.1% Ag as additive, TiO₂ with 0.3% Ag, and TiO₂ with 0.6% Ag as additive. A plain glass slide, not coated with any nanoparticle was used as a control. About 100 µL of the suspension was added this plain glass slide also. All these glass slides were incubated at 37°C in visible light. After one hour, all these suspensions are washed with 100 µL distilled water and 100 µL was taken from each of these emulsions and inoculated onto sterile Mueller Hinton Agar (MHA) plates. All these MHA plates were incubated in the incubator at 37°C overnight. The slides were placed back in the visible light. After every one hour interval for a total of eight hours, the same method was followed and each time the slides were washed with 100 µL distilled water and 100 µL of the washed suspension was inoculated onto fresh sterile MHA plates by streak method and incubated overnight in the incubator at 37°C. The Colony Forming Unit (CFU) was counted individually on a colony counter in each of these plates after overnight incubation [Table/Fig-1].

RESULTS

At the start of the experiment i.e., at 0 hours, all the 25 samples which were added to all the slides containing nanoparticles showed >1x10⁸ cfu/ml. However, there was considerable reduction in the colony forming units on all the nanoparticle coated slides over time.



[Table/Fig-3]: Growth of *E. coli* treated with TiO₂ and TiO₂ with silver as additives at 8 hours of incubation. a: control, b: TiO₂ annealed at 200°C, c: TiO₂ annealed at 400°C, d: TiO₂ with 0.1% Ag, e: TiO₂ with 0.3% Ag, f: TiO₂ with 0.6% Ag



[Table/Fig-4]: Line graph showing rate of reduction of the cfu/mL of *E. coli* over time at different nanoparticle concentrations.

The organisms which were inoculated onto TiO₂ annealed at 200°C showed a slower reduction rate, reducing from >1 × 10⁸ cfu/ml to <1 × 10³ cfu/ml after six hours of incubation in visible light but the total absence of organisms was observed only after eight hours of incubation. However, in control slide, which was not treated with any of the nanoparticles, there was a growth of >1 × 10⁸ cfu/ml even after 24 hours of incubation.

There was a considerable reduction in the cfu/ml in the TiO₂ annealed at 400 °C from >1 × 10⁸ cfu/ml to no growth after six hours of incubation, which was a slight improvement from the TiO₂ annealed at 200°C. In plates with organism inoculated onto 0.1% Ag showed a decrease of cfu/ml to <1000 cfu/ml after four hours treatment and to no growth after six hours.

The organisms inoculated onto TiO₂ coated slide with 0.3% Ag as additive showed a reduction of cfu/ml to <1 × 10³ within three hours of growth itself which was faster than TiO₂ with 0.1% Ag, while at the same time, there was no growth of any bacteria with TiO₂ with 0.6% Ag as additive [Table/Fig-2]. At eight hours of incubation, except in the control plate, there was no growth in any of the other plates [Table/Fig-3]. [Table/Fig-4] shows that though TiO₂ in its pure form was highly antibacterial, the antibacterial activity increased with the addition of silver and with the increase of the concentration of silver, this activity further increased.

DISCUSSION

The photocatalytic activity and the antibacterial properties of TiO₂ have been improved by doping the surface with transitional metal ions and organic polymers. It is well demonstrated that the photocatalytic reactions are initiated by the most important trigger of light absorption that relates directly to the band gap between the valence and the conduction of TiO₂ crystals. The photocatalytic reaction can be justified by the following processes. Firstly, light absorption takes place followed by excitation of electrons (with in nano seconds) from the valence band of TiO₂ to the conduction band to form electron/hole pairs, this is preceded by migration of electron/hole pairs thus formed to the TiO₂ surface. Secondly, initiation of, redox reactions take place with the hole of the valence reacting with H₂O or hydroxide ions which are adsorbed on to the surface and lead to the formation of hydroxyl radicals as well as superoxide ions. These hydroxyl ions are very powerful and are indiscriminate oxidizing agents against the bacteria [16].

It has been suggested by Zhang Y et al., that the metal oxides consist of a positive charge while the microorganisms exhibit a negative charge, which is the reason of the electromagnetic effect between the organisms and the metals, leading to the oxidation of the organisms, ultimately resulting in the death [17]. However, Sangchay W et al., in his study has reported that be the photocatalytic effect of TiO₂ causing the hydroxyl radical attack and lipid peroxidation reaction. The silver ions increase the oxygen anion radicals and as

a result causing reactive centre in the TiO₂ surface. With water, this photogenerated holes can react and form hydroxyl radicals [18].

In the present study, we have observed the antibacterial effect of pure TiO₂ annealed at 200°C and 400°C and TiO₂ doped with 0.1% Ag, 0.3% Ag and 0.6% Ag on drug sensitive *E. coli* when incubated in visible light. Our study showed a steady decrease of colony forming units/ml from 0 hours to eight hours. At 0 hours, as the stock solution was adjusted to 0.5 McFarland's constant, all the plates had a growth of around 1.5 × 10⁸ cfu/ml. At three hours, there was a steady decrease to 10³ cfu/ml in all the conditions. By eight hour interval, there was no cfu/ml on all the nanoparticle coated glass slide while the one on control slide showed confluent growth (>1 × 10⁸ cfu/ml). In the TiO₂ annealed at 200°C, the number of colony forming units reduced to <1 × 10³ cfu/ml at sixth hour interval while in TiO₂ annealed at 400°C, there was no growth at sixth hour interval. However, in the slide containing TiO₂ doped with 0.6% wt Ag, there was a slight contamination due to mishandling of the slides at sixth hour interval [Table/Fig-2].

In a study by Sangchay W et al., silver doped TiO₂ thin films eliminated *E. coli* much faster than TiO₂ alone even under UV radiation [19]. These results were in accordance to our study, where TiO₂ doped with Ag were much more effective than only pure TiO₂. With increasing concentrations of silver, the antibacterial effect was faster. In a similar study by Sophee S et al., a reduction of the colony forming units/ml in the ATCC strains of *E. coli* when treated with TiO₂ with increased concentrations of zinc oxide as the additive. Similar results were observed when incubated both in visible and UV light showing that zinc oxide also had similar antibacterial effect as silver [20].

Efficacy of TiO₂ as a topical agent as well as in combination therapy was analyzed by other researchers in their study and reported that an increase in the antibacterial effect was associated with the increase in the concentration of TiO₂ [21].

Silver as additive for antibacterial effect was also tested on other ATCC strains of bacterial pathogens such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, with similar results [12]. Several investigators have reported oxidative damage to cell membrane or internal cell structures with or without illumination [20,22].

With the emergence of multiresistant bacteria, there is an urgent need for newer techniques that can efficiently kill them. Nanoparticles have offered a new strategy to tackle these bacteria. It has been reported that four types of silver carbon complexes with different formulations are effective against many such medically important bacteria such as *Pseudomonas*, *Acinetobacter*, Methicillin Resistant *Staphylococcus aureus* (MRSA) [3].

Most of the studies studied earlier were done on water samples or on ATCC strains [12,18-22]. The present study was one of the very few studies which used actual clinical samples and human pathogens instead of ATCC strains. It was observed that the antibacterial effect of the nanoparticles on clinical samples was similar to that on ATCC strains. This study has opened the path to venture the efficacy of the nanoparticle coated implants on the infectious human pathogens. Future studies need to be performed in vivo to see the effects of nanoparticles of TiO₂ with or without Ag within the body after the implantation of the biomedical device.

LIMITATION

However, there are few limitations of the present study. This study was performed only on non Extended Spectrum of Beta Lactamases (ESBL) *Escherichia coli* isolated from the clinical samples. The sample size was small with only 25 samples of *E. coli*. Further tests need to be performed on multi drug resistant *E. coli* such as ESBLs as well as on other coliforms and bacterial pathogens. There are other additives such as copper, zinc that can be added to TiO₂. Comparison of the efficacy of antibacterial effect of silver and the other additives can also be performed.

CONCLUSION

TiO₂ is found to be antibacterial and thus can be used as disinfectant. When doped with silver, the antibacterial effect was increased. Thus, efficiency of nanoparticles as biocide agent can be increased doping with some other metal oxides. The research on biomedical applications of nanoparticles has only recently begun. Further research can be conducted by coating nanoparticles onto biomedical appliances such as scalpels and other implants to assess their efficacy in vivo.

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