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RESEARCH ARTICLE

ANTIMICROBIAL EFFECT OF SYNTHESIZED TiO_2 NANOPARTICLES AGAINST PATHOGENIC MICROORGANISMS

Upasana Yadav[1], B. C. Yadav[2], Manodeep Sen[3], Tanushri Chatterji[2] and Qamar Rehman[1]

[1]Department of Life sciences, Amity School of Applied Sciences, Amity University, Lucknow-226017, U.P., India
[2]Department of Applied Physics, School for Physical Sciences, BabasahebBhimrao Ambedkar University, Lucknow-226025, U.P., India
[3]Department of Microbiology, Dr R. M.L. Institute of Medical Sciences, Lucknow, U.P., India

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ABSTRACT

Since many investigations into antimicrobial activity of titanium oxide have been published it still remains a particularly active research field. To our knowledge, the bactericidal properties of TiO_2 synthesized by ball milling method have not been tested extensively. In this connection we synthesized TiO_2 by ball milling method by using high-purity (99.9%) elemental powder of Ti of ~ 0.5 mm size. The powder was mechanically milled in a high-energy a ttritor ball mill (Szegvari Attritor) and examined its antibacterial properties. Antimicrobial action of as-prepared TiO_2 was investigated using *E. coli, P. aeruginosa* and *S. aureus*. The bacterial growth was examined in the presence of TiO_2, by the effect of UV light, and in the presence of both-TiO_2 and UV light. The dynamics of antimicrobial action of the selected preparations was assessed by killing curves determination. The efficiency of antibacterial activity in standard experimental conditions as function of time was discussed. It can be concluded that the synthesized TiO_2 (anatase) possesses strong antibacterial activity and could be used effectively for disinfection under UV illumination.

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INTRODUCTION

Nanotechnology is the study of particles of size of 0.1 to 100 nm. Nanomedicine is a relatively new field of science and technology. Nanomaterials refer to materials with special properties, whose geometric dimension reaches to Nanoscale. Nanoparticles have a high fraction of surface atoms and a high specific surface area; have been studied extensively because of their unique physicochemical characteristics including electronic properties, optical properties, catalytic activity, antibacterial properties and magnetic properties [1-10]. An emerging field of Nano science is Nanobiotechnology which utilizes Nano based-systems for various biomedical applications [11]. Nanotechnologies have various applications in medical field such as to enhance the performance of medical devices, to produce diagnostic imaging materials and in novel drug delivery systems, [12] Microbial resistance to antibiotics is a world-wide problem in humans and animals. An extensive use of antibiotics is generally accepted that the main risk factor for the increase in the antibiotic resistance. This has led to the emergence and dissemination of resistant bacteria and resistance genes in animals and humans [13]. Thus current challenge is the discovery of new antimicrobial agents against resistant bacterial strains produced by different mechanisms [14,15]. Resistance among bacteria is continuously increasing. Nanotechnology by using atomic scale tailoring of materials is expected to open new avenues to fight and prevent diseases. Recently it has been demonstrated that metal oxide nanoparticles exhibit excellent biocidal and biostatic action against Gram-positive and Gram-negative bacteria [16]. Antimicrobial agents are highly relevant for a host of industrial applications in environmental, health care, food, synthetic textiles, packaging , and medical care products. Metal oxide nanoparticles (NPs) are known to possess strong antimicrobial properties[17].

Naturally occurring oxide of Titanium dioxide, is also known as titania. Various properties of titanium dioxide are high refractive index, light absorption, non-toxicity, chemical stability, optical, dielectric property, relatively low-cost production and photo-catalytic properties from size quantization

*✉ ***Corresponding author:*** **Upasana Yadav**
Department of Life sciences, Amity School of Applied Sciences, Amity University, Lucknow-226017, U.P., India

[18-22]. Titanium dioxide nanoparticles have attracted attention in the fields of environmental purification, solar energy cells, photocatalysts, gas sensors, photo electrodes and electronic, as a self-cleaning and self-disinfecting material for surface coating in many applications. TiO_2 can also widely used as a pigment in ointments, toothpaste, paints, etc [23-27]. By the crystalline structure, the morphology and the size of the particles the performances of TiO_2 are strongly influenced [28-33]. Nanosized TiO_2 particles are of particular interest due to their specifically size-related properties. Changes in pressure and heat causes TiO_2 transitions between various phases .Among various phases of TiO_2 anatase shows a better photocatalytic activity and antibacterial properties [34-38]. Anatase has attracted much attention owing to its application in photovoltaic cells [39] and as photocatalyst. Titanium dioxide is widely used these days as self-cleaning, self-disinfecting and surface coating materials [40]. Titaniumdioxide has a more helpful role in environmental purification due to its photo induced super-hydrophobicity and antifogging effect [41]. Some antimicrobial agents are extremely irritant and toxic and current researches are focused on formulate new antimicrobial agent without any toxic effect.

For photocatalytic application titanium oxide powder can be synthesized by variety of techniques including sol-gel method, solvothermal process, reverse micellar, hydrothermal method, ball milling, plasma evaporation, etc.[42-43]. For synthesis of metal oxide nanoparticles offer advantages such as high crystallinity at low temperatures, robust synthesis parameters and ability to control the crystal growth [44-45]. Anatase TiO_2nanoparticle was synthesized by nonhydrolytic sol-gel reaction and studied its photocatalytic activity toward degradation of phenol. To our knowledge, the bactericidal activity of TiO_2 performed by ball milling method still has not been tested extensively. For this reason we studied the antimicrobial action of ball milling method synthesized TiO_2 against *E. coli, P. aeruginosa*and *S. aureus*as representative species of Gram-negative and Grampositive bacteria.

MATERIALS AND METHODS

Materials

All reagents used in the synthesis of TiO_2 and antibacterial studies were analytical grade and employed without any further treatments. Distilled water was used for all synthesis and antimicrobial activity processes. *Esherichia coli* (ATCC 8739), *Stapyloccusaureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 36232) and Bacillus subtilis (ATCC 6633) were obtained from American Type Culture Collection (ATCC). All the media and chemicals were purchased from Oxoid Microbiology Products and Difco Laboratories.

Ball milling method for synthesis of Tio_2 nanoparticle

TiO_2 was synthesized by using high-purity (99.9%) elemental powder of Ti of ~ 0.5 mm size. The powder was mechanically milled in a high-energy attritor ball mill (SzegvariAttritor). The attritor has a cylindrical stainless steel tank of inner diameter 13 cm. The grinding balls made of stainless steel are of 6 mm in diameter. Ball milling was carried out for 10 hrs at 400 rpm with ball to powder ratio of 40:1 for preparing TiO_2 nanoparticles. The pelletization of the ball milled powder was done using hydraulic press machine (MB Instrument, Delhi) under a pressure of 616 MPa at room temperature. [46]

Antibacterial Activity: Experimental Setup for Bacterial Cultures

ATCC bacterial strains of *Escherichia coli* (ATCC 35282), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (ATCC 27853) were taken as reference for sensitivity testing against nanoparticle titanium oxide (TiO_2). Standard microbial strains (ATCC) were isolated on Blood Agar Plate (BAP) and Mac Conkey Agar Plates followed by 24 hours of incubation at 37^0 C, one colony of each strain was inoculated in Tryptic Soy Broth (TSB). Further, 100 µl of inoculums was added to 10 ml phosphate buffer saline which was standardized to 0.5 McFarland. Antibacterial activity was measured using 4 samples solutions: (i) Inoculum as control (ii) Inoculum + UV rays for 20minutes (iii) Inoculum + TiO_2 (10 µl/50 ml concentration) (iv Inoculum + $TiO_{2\,+}$ UV rays. The prepared inoculums were further used for sensitivity testing on Mueller Hinton Agar Plate (MHA) plates by spread plate technique. The antibacterial activity was further followed by colony forming unit (cfu) count per plate.

Killing curves

The dynamic of antimicrobial action was assessed by killing curves prepared by time-killexperiments. At regular interval of time (5, 10 or 15 min, respectively) 500 µl fromexperimental flask were withdrawn and serial dilutions were prepared before plating. The numberof viable cells was determined by spread plate method as following: 100 µl from any dilution werestreaked in duplicate on Mueller-Hinton agar (MHA), and poured in Petri dishes (Ø=90 mm) with agar depth of 5 mm. After overnightincubation at 37^0C the colonies on the plates were counted and results were calculated to 1 ml.Time killing curves were performed by plotting mean colony counts (CFU/ml) versus time in minutes. All the protocol was followed in sterile condition and culture and plating was performed in laminar air flow cabinet.

RESULT AND DISCUSSION

Characterization of tio_2 nanoparticle

X-Ray Diffraction

From the analysis of XRD data figure(1) it is clear that all the peaks are of the rutile phase, no other phase was identified.

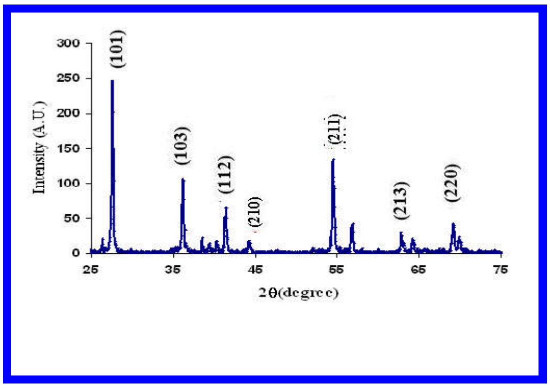

Fig 1 X-ray Diffraction pattern of TiO_2 in the form of powder.

It can be observed that there are sharp diffraction peaks which indicate that the sample has crystalline nature and no amorphous phase has been formed during present milling conditions. This result suggested that the nano-TiO_2 powder is composed of irregular polycrystalline material

Transmission Electron Microscopy

The TiO_2 particles arehighly strained due to the ball milling and it can be seen in form of intergrowth in Figure 2. The size distribution of nanoparticle has been observed between 10-20 nm. The corresponding selected area diffraction pattern in Figure 2 below show a spotty rings, it is due to the nano crystalline nature of TiO_2 particles.

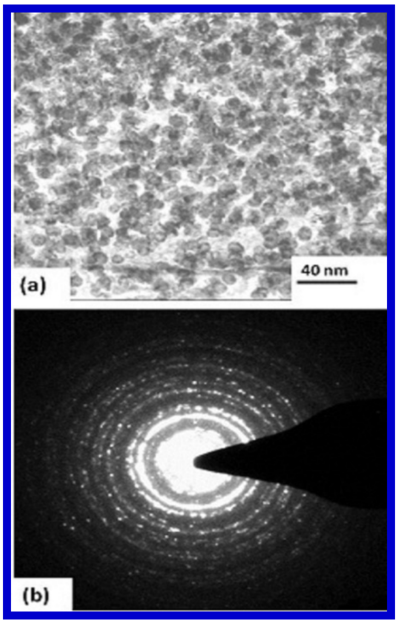

Fig 2 TEM of TiO_2in the form of powder.

UV-visible Spectroscopy

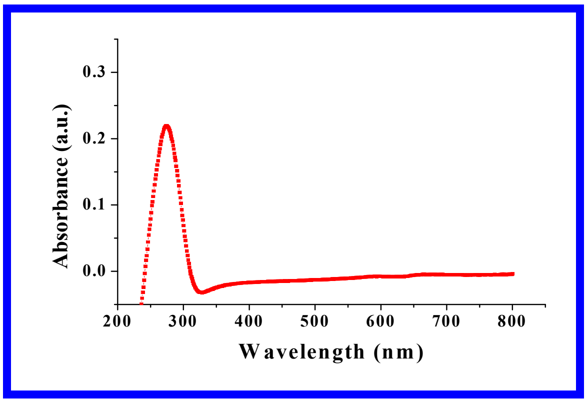

Fig 3 UV-Visible absorption spectra of TiO_2material

Optical characterization of the sensing material was done by UV-visible spectrophotometer (Varian, Carry-50Bio). Figure 3 shows UV-visible absorption spectra of titanium oxide in UV and visible range. Titanium oxide nanoparticles reveal a strong change of their optical absorption when their size is reduced. Therefore, absorption spectra of titanium oxide nanoparticles obtained in the UV-visible region show blue shift in the absorption edge at 268 nm in comparison to bulk titanium oxide. The corresponding band gap was found 4.46 eV respectively. It is evident that titanium oxide shows significant blue shift of the absorption peak relative to the bulk absorption. This blue shift is useful for gas sensing applications.

Antibacterial action of TiO_2 against Microbial Strains

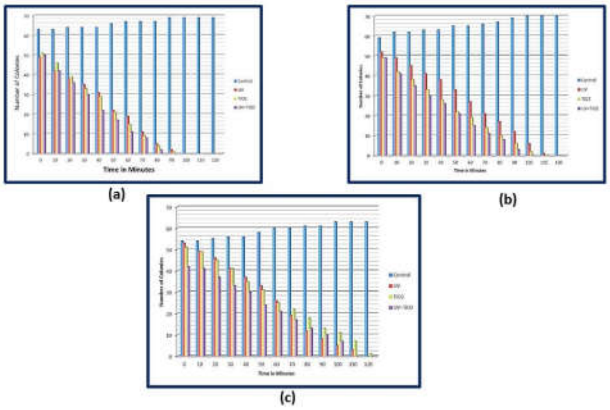

Fig. 4 Graphical Representation for *(a) E.coli(b) Pseudomonas aeruginosa(c) Staph aureus*

Time kill curves at different experimental conditions.

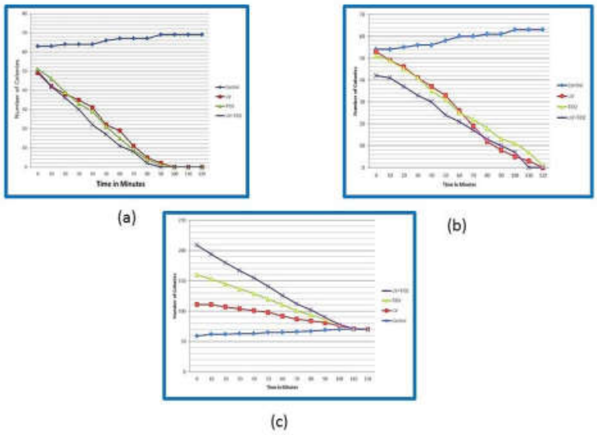

Fig.5 Time kill curves at different experimental conditions. *(a) E.coli (b) Staph aureus (c) Pseudomonas aeruginosa*

The sequences order of sensitivity of TiO_2 photocatalytic action against microbial strains is as following: *E. coli*>*P. aeruginosa*>*S. aureus*. Most effective antibacterial activity was observed in TiO_2+UV rays against *E.coli* when compared to the control. The antibacterial properties of the strains were observed after 2 hours at 10 minutes of interval. Although the bactericidal property of UV Rays, TiO_2, UV+TiO_2 was quite remarkable after 90 minutes as less than zero or zero colony count was noticed against the three bacteria strains. The graph plot obtained in the parameters of time in minute (X-axis) to No. of colonies (Y-axis) is shown in Figure 4 and Time kill curves at various experimental conditions for E. Coli, Staph aureus and Pseudomonas aeruginosa are plotted as Fig.5 (a), (b) and (c).

Our results are in agreement with usually 16 reported order of bacterial susceptibility in photoinactivation processes with titanium oxide: *E. coli*> Gram-negative bacteria (other than E.coli) > Enterococcus species > Gram-positive bacteria [31].

This arrangement might be explained with the specific structure of the bacterial cell wall. Grampositive bacteria have a thick cell wall, containing many layers of peptidoglycan and teichoic acids. The cell wall of Gram-negative bacteria is a relatively thin, but it possesses an additional outer membrane containing lipopolysaccharides and lipoproteins bilayers. Gram-positive bacteria have been documented to be photocatalytically more resistant than Gram-negative. As a result, a higher number of hydroxyl radical attacks for Gram-positive bacteria are needed to get the complete bacterial inactivation [12, 25, 28, 38, 45].

CONCLUSION

Anatase titanium dioxide nanoparticles have been synthesized by ball milling technique. These nanoparticles have a particle size of 20 nm and its antibacterial properties were tested in standard conditions. Our experimental setup allows comparing the antimicrobial activity of TiO_2 alone, and in presence of ultra violet radiation against different kind of bacteria in equal initial cells concentration. As-prepared TiO_2 shows significant antibacterial activity under UVA radiation. It was very effective in removing of test-microorganisms and in concentration of 1 mg/ml effectively kills Gram-positive and Gram-negative bacteria within 35 minutes. It could be concluded that the synthesized TiO_2 possesses strong antibacterial activity against the important human pathogens tested and could be used successfully for disinfection under UVA illumination.

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