Effect of hydrothermal time and acid-washing on the antibacterial activity of Sodium titanate nanotubes

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Abstract. Bacterial resistance to antibiotics has been a major concern globally. In this work, sodium and hydrogen titanate nanotubes were successfully synthesised using a facile hydrothermal technique. The prepared samples were characterized by XRD, FTIR, HRTEM surface area analyser, hydrodynamic size analyser and zeta potential. Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) measurements besides agar well diffusion method showed good antimicrobial activity of both nanotubes. The antimicrobial activity of sodium titanate nanotubes prepared at 6 and 23 hr., and their hydrogen exchanged forms; (HTNT) and (HTNS) explored good antimicrobial activity against both Gram positive and Gram negative bacteria besides their antifungal activity which reflects their importance in treatment of bacterial infections causing serious diseases in both animal and human.

1. Introduction
Nanotechnology has emerged as one of the most useful tools for facing numerous biological and biomedical challenges [1]. Nanoparticles can offer enhanced properties and better results due to their higher surface to volume ratio compared to bulk materials. Nanoparticles have been shown to possess improved chemical, mechanical, electrical, optical and magnetic properties [2]. One of most promising biomedical application of nanomaterials is their use as antibacterial and antimicrobial agents for drug resistive pathogens (DRPs). Resistance to antibiotics is considered a big challenge in pharmaceutical and biomedical fields of study [3]. DRPs represents a serious threat for containing their spread and for their serious impact on human health [4,5]. Patients infected with DRPs are susceptible to increased treatment costs, longer treatment periods in hospitals and require the use of 2-3 different antibiotics which is a less effective and more toxic technique [3,4]. Studies have shown that multi-drug resistive bacteria has caused thousands of deaths and novel methods and strategies are of urgent need [5].

Several types of nanoparticles have shown great promise as antibacterial agents compared to standard antibiotics [6–9]. Silver [10], ZnO [11], TiO2[12,13] and chitosan [14] nanoparticles are among the most heavily studies nanomaterials as antibacterial agents. Titanium dioxide and titanate nanostructures offer several advantages such as being cheap, available, ion exchangeable and easy to synthesize in different morphologies[15]. Several studies available in the open literature reported promising results for the use of titanate and modified titanate nanomaterials for antimicrobial
applications. Yada et al modified the surface of a sodium titanate nanofiber with silver and utilized it as antibacterial agent against Staphylococcus aureus[16]. Results showed that silver exchanged titanate thin films showed promising antibacterial effect against the tested microorganism. Lee et al reported that silver exchanged titanate structures are very favourable materials for antibacterial applications [17]. Matsushita et al. have showed that calcium modified titanate films on top of Ti metal structures show strong antibacterial properties against the tested Staphylococcus aureus microorganism [19]. Stoyanova et al. studied the antimicrobial effect of Zinc titanate nanostructures prepared by sol-gel technique and reported promising results against Esherihia coli [18]. Moreover, Shah et al reported significant results against P. aeruginosa and S. aureus when using barium titanate nanoparticles as novel antibacterial powder [19]. To this extent, no study has reported the effect of hydrothermal time and acid washing on their antibacterial and antimicrobial activities against pathogens. The aim of the current work is synthesized and characterize sodium titanate nanotubes at different hydrothermal times and also their hydrogen exchanged nanotubes. The prepared samples are to be tested as antimicrobial agents against several pathogens including P. Aeruginosa, E. Coli, Staphylococcus and Streptococcus.

2. Materials and methods

2.1. Materials
Titanium dioxide, sodium hydroxide and Hydrochloric acid were supplied by Loba chemie, Piochem for laboratory chemicals and Adwic respectively. All chemicals were used as received without further purification.

2.2. Synthesis of sodium and hydrogen titanate nanotubes
Sodium titanate nanotubes(STNS) were prepared following a facile hydrothermal technique as previously reported in our previous work [20–22]. Briefly, 10 g of TiO2 are suspended in 500 mL of 10 M NaOH solution. The solution is kept under vigorous stirring for 30 min. Then the solution is transferred to a 1L stainless steel autoclave which was heated at 160 °C for 6 and 23 hours to form STNS and STNT respectively. The autoclave was then left to cool naturally and the formed powder was then separated and washed extensively with distilled water then dried at 80 °C overnight. The Sodium ions replacement process was conducted by washing STNT or STNS with 0.1M HCl solution, where the mixture was subjected to sonication for 30 min. using ultrasonic with probe (20 KHz).

2.3. Characterization
X-ray diffraction experiments were conducted on a PANalytical (Empyrean) X-ray diffraction using Cu Kα radiation (wave length 0.154 cm⁻¹) at an accelerating voltage 40 KV, current of 35 mA, scan angle range from 20 to 70° and scan step 0:02°. The BET specific surface area, specific pore volume, and pore sizes of the adsorbent materials were determined by N2 adsorption isotherms using an automatic surface analyzer (TriStar II 3020, Micromeritics, USA). Hydrodynamic particle sizes and zeta potentials were determined on a Malvern zetasizer instrument (Malvern Instruments Ltd).

2.4. Isolation and identification of bacterial pathogens

2.4.1. Isolation of bacterial pathogens. E. coli strain was grown on its selective media MacConkey agar media (Oxoid; CM 0115) and Eosin Methylene Blue agar (EMB; Oxoid; CM 69) plates whereas, the other strains (Staphylococcus and Streptococcus) were determined on agar plates (Gram stain and colony morphology). The plates were incubated at 37°C for 48 hours and the bacterial growth was observed for all of the isolates[23].
2.4.2. Biochemical and serological identification of bacterial pathogens. Standard Kits (Biomerieux, Marcy L’etoil, France) API had been used for both physiological and biochemical identification of each bacterial isolates of both gram positive and negative bacteria.

2.5. Bacterial strains and culture conditions
For antibacterial studies, Gram-positive St. coccus, S. aureus and Gram-negative E. coli and P. Aeruginosa were four microbial agents of multiple infective diseases in humans and animals, chosen as the target organism for this investigation. E. coli (ATCC 25922), S. aureus (ATCC 25913), St. coccus (ATCC 49619), and P. aeruginosa (ATCC 27853) were purchased from Cairo Microbiology Research Centre besides two different strains of fungi species (Aspegillus Flocculosus and Aspegillus nigricans). Sabouraud 2% Glucose Agar with Chloramphenicol media for microbiology was used for fungal culturing before start working. All tubes were sterilized in an autoclave before the experiments. Muller Hinton broth was used to culture E. coli, S. aureus, St. coccus and P. aeruginosa at 37 °C for 24 hour in an incubator. The liquid cultures were finally diluted to obtain bacterial cell concentration of approximately 10⁷ colony forming units (CFU)/ml for following antibacterial test.

2.6. Determination of minimum inhibition concentration (MIC)
Using broth dilution method according to Clinical Laboratory Standards Institute (CLSI)[24]: The MIC was defined as the lowest concentration that completely inhibited the growth of microorganisms for 24 hours. Bacterial strains were grown overnight on MHA plates at 37°C before being used. The antimicrobial activity of the obtained nanotubes was examined using the standard broth dilution method [24].

For each sample, different concentrations were diluted with Muller Hinton broth to give a final concentration ranging from (1000 μg/ml-6.25 μg/ml) in the presence of both positive and negative control tube. The bacterial isolate was sub cultured on Muller Hinton Agar (M.H.A) and incubated at 37°C for 24 h [25]. Colony from the tested microorganism was suspended in 5ml saline, and the suspension was adjusted to 0.5 McFarland standard to give organism suspension of (1×10⁸ CFU/ml). All tubes were incubated at 37°C for 24 hrs. Results were recorded in terms of MIC, which is the lowest concentration of antimicrobial agent causing almost complete inhibition of growth or giving no visible growth.

2.7. Minimal bactericidal concentration (MBC)
After MIC determination of the nanoparticles tested, aliquots of 100 μl from all tubes in which no visible bacterial growth was observed were seeded in MHA plates and were incubated for 24h at 37°C. The MBC endpoint is defined as the lowest concentration of antimicrobial agent that kills 100% of the initial bacterial population. The number of plates without colonies was noted [24].

The MIC of STNT, STNS, HTNT and HTNS was measured by broth dilution test in dark condition. The tubes were incubated at 37°C for 48 h on a rotary platform. The visual turbidity of the tubes was noted before and after incubation. The MIC was defined as the endpoint where no visible turbidity could be detected.

2.8. Antibacterial Sensitivity of the Synthesized Nanomaterials
Each tested Nanomaterials was prepared to give a final stock concentration of 10 mg/ml. This stock solution was used for preparing different diluted concentrations of each material in distilled water. The antibacterial activity of the synthesized nanomaterials was measured using agar well diffusion method in presence of Difloxacin 92.9% as a standard reference drug to Gram negative bacteria as E-coli and Tylvalosin to Gram positive bacteria as S. aureus.

Agar diffusion method is one of the formal techniques, in this test; the tested samples were spread on agar layer in a Petri-dish. The growth of the tested microorganisms was limited to a circular area or zone around the pores which contained tested material. The antimicrobial effects were determined and measured as a diameter zone in millimetres by using calibre.
The experimental procedures were as follows: The bacterial strains were suspended in saline solution with an intensity of $1.5 \times 10^8$ viable organisms/mL, the population of the investigated pathogen was matched to McFarland tube No. 0.5 ($1.5 \times 10^8$) colony forming units CFU/mL. Standard sterilized discs were impregnated overnight with the three different tested concentrations of the tested nanomaterials using standard size (50 mm diameter) of Whatman filter paper. The impregnated discs were placed aseptically with some sterile forceps on Mueller-Hinton agar plates. All plates were incubated at 37°C for 24 hr, then inhibition zone diameter of all tested bacteria was determined via two-fold serial dilution. All the readings were taken in triplicate.

3. Results and discussion

3.1. Samples characterization

The powder XRD diffractograms for all prepared samples are shown in Figure 1. The diffractograms of both samples HTNS and HTNT were matched with the standard JCPDS 47-0561 file. The diffraction peaks at 24.6, 25.6, 28.4, 38.1 and 48.7 were indexed to the (110), (202), (111), (60-2) and (114) planes. The STNT and STNS samples were indexed in reference to the ICDD-04-0157486 standard data. The peaks at 10.2, 24.1, 25.5, 28.7 and 48.2 could be indexed to the (001), (201), (011), (111) and (303) planes. The average crystallite sizes of STNT, HTNT, STNS and HTNS were calculated to be 31.86, 31.87, 63.82 and 63.84 nm respectively. As expected, no significant change in the average crystallite size was caused by hydrogen replacement. FTIR spectra of all prepared samples is shown in figure 2. The peaks at 3400 cm$^{-1}$ and 1620 cm$^{-1}$ can be attributed to the stretching vibrations and deformation of hydroxyl groups of physically adsorbed water molecules [28]. The low intensity peaks are typical of the Ti-O and Ti-O-Ti bonds [29].

![Figure 1. Powder XRD diffractograms of STNT, HTNT, STNS and HTNS.](image)

![Figure 2. FTIR spectra of all prepared samples.](image)

Figure 3 shows the HRTEM images of the prepared titanates, it is clear from that the sodium titanate nanotubes were successfully prepared at 6 and 23 hr. The HRTEM images reveal that increasing the hydrothermal time from 6 to 23 hr enhanced the quality of the prepared nanotubes, where the sample obtained at 6 hr contains some unrolled nanosheets. After acid-washing no significant change occurred in the morphology of the samples. It is clear that the average diameter of the prepared nanotubes is lower than 20 nm, while the average length is lower than 1000 nm in all samples.
Figure 3. HRTEM image of (a) STNS, (b) STNT, (c) HTNS, and (d) HTNT

The BET surface area for all prepared samples was estimated using Nitrogen adsorption isotherms as shown in figure 4 (a). The BET surface area of STNT, HTNT, STNS and HTNS were calculated to be 114.57, 141.84, 96.48 and 135.9 $\text{m}^2/\text{g}$ respectively. For all samples the isotherm follows a type IV behaviour indicating the presence of mesoporous pores within all the samples [24]. The pore size distribution of the samples is shown in figure 4 (b). The pores present are probably interparticle pores formed due to the aggregation of the nanomaterials as no surfactant is used during preparation.

Zeta potential for all samples is tabulated in Table 1. STNT and STNS showed a zeta potential of -11.2 and -11.7 mV respectively. Sodium titanate based structures had close zeta potential values irrespective of the morphology indicating the role of sodium ion on the overall surface charge. When sodium was replaced with hydrogen, HTNT and HTNS showed a zeta potential of 2.5 and 0.13 mV respectively. The hydrodynamic sizes for all samples are shown in Table 1. The large values reflect the extensive agglomeration of the particles in solution owing to their low zeta potential values. Another plausible reason for the large values is the particle shape (tubes and/or sheets).
3.2. Antimicrobial study

In this work, the antimicrobial effect of STNT, HTNT, STNS and HTNS was investigated. Titanate nanostructures are known to have promising antimicrobial potential under photocatalytic conditions [26]. In this work all samples were tested under dark conditions. As shown in figures 6 and 7, all samples showed antimicrobial activity against the tested pathogens. Sodium titanate nanotubes (STNT) in this study showed the best antimicrobial activity than other prepared shapes, even though slightly higher MIC than other shapes except in pseudomonas infection; showed also excellent antimicrobial activity (MIC) but that’s due to the visual accuracy for the MIC evaluation while the actual antimicrobial activity achieved through the disc diffusion method. Only few studies in the literature reported antimicrobial activity for titanate nanostructures under dark conditions. Hydrogen titanate nanofibers and nanotubes possess significant antibacterial activity against Gram-positive bacteria and also against Gram negative bacteria (E. Coli) but in lower activity than the positive one according to the measured zone of inhibition when compared to the Gram positive one as also mentioned by Kundu et al. [27]. Neagu et al. [28] also showed that hydrogen titanate nanotubes has a significant antibacterial effect against Bacillus subtilis and Virgibacillus halodenitrificans which may be due to the interaction of the nanotubes (synthesized at different conditions) with the cell wall composition of the microorganism. The results reported in this study shows that the activity of hydrogen titanate nanostructure (HTNT and HTNS) against Gram-positive bacteria is not always better than those for Gram-negative ones as opposed to results by Kundu et al. [27]. It can be observed from figure 5 that the MIC of both HTNT and HTNS is much lower in case of Escherichia coli compared to Streptococcus Pneumonaie and Staphylococcus aureus. It can be also shown that the MIC for Escherichia coli is significantly lower than that measured for Pseudomonas aeruginosa which suggests that the antibacterial effect depends on the interaction between the

| Zeta potential (mV) | Hydrodynamic size (nm) |
|---------------------|------------------------|
| STNT    -11.2    | 1704                     |
| HTNT    2.5       | 1822                     |
| STNS    -11.7    | 1758                     |
| HTNS    0.13     | 1686                     |
nanostructure and the cell wall of the microorganism as reported by Neagu et al. [28] even for Gram-negative strains.

Sodium titanate nanotubes possess an antibacterial effect against methicillin-resistant Staphylococcus aureus (MRSA) Yada et al. [16,29]. The authors showed that the tested nanotubes have similar antibacterial effect in dark as that under UV illumination. The results in our study confirm the antibacterial effect of sodium titanate nanostructures (STNT and STNS) against both Gram-negative and Gram-positive bacteria. As illustrated in Figure 5, both structures showed MIC values comparable to both HTNT and HTNS for the Gram-negative and Gram-positive bacteria strains tested. This shows that one of the main factors defining the difference between sodium and hydrogen based titanate nanostructures is their zeta potential. For the hydrogen based titanate nanostructures the zeta potential and hence the surface charge was insignificantly different as previously discussed. Similarly, sodium and based titanate nanostructures showed close zeta potential values. This illustrates that zeta potential is the main driving factor behind the interaction between the nanostructures and the bacteria cell wall. Irrespective of the surface area and the crystallite size, the surface charge will affect the electrostatic attraction or repulsion between the surface of the nanostructures and the bacteria cell wall. This will control the nature of interaction (bacteria – nanoparticles) and hence the MIC for each bacteria strain. Finally, STNT is the best and the more efficient one in the treatment of both Gram positive and Gram negative bacteria.

(a) Minimum inhibitory concentration
(b) Minimum bactericidal concentration

Figure 5. MIC (a) and MBC (b) of STNT, STNS, HTNT and HTNS. MIC was determined by micro broth dilution technique and values reported are the values (Mean) obtained in triplicate.
Figure 6. Zone of inhibition of STNT (1), STNS (2), HTNT (3) and HTNS (4) against both Gram positive and Gram negative bacteria.

(1) Equal to 1000 μg/ml; (2) 500 μg/ml; (3) 250 μg/ml; (4) 125 μg/ml

Figure 7. The actual measured zone of inhibition of STNT, STNS, HTNT and HTNS against both Gram positive and Gram negative bacteria.

4. Conclusion
A facile hydrothermal technique was used to prepare sodium and hydrogen based titanate nanotubes. The crystalline structure of the prepared samples was verified by powder XRD diffraction analysis. Samples were successfully characterized by surface area analyser, hydrodynamic size analyser and zeta potential. The antibacterial activity of STNT, STNS, HTNT and HTNS on E Coli, S.aures, St.coccus, and P. aeruginosa besides, two different strains of fungi (Aspegillus Flocculosus and Aspegillus nigricans) was investigated. The experimental results indicated that STNT, STNS, HTNT and HTNS have an important antibacterial effect against different microorganisms causing serious infectious diseases in human and animals. To summarize, the resistant bacterial strains to antibiotics have become a serious public health problem, so increasing the demand for discovering or development new antibacterial materials is a strong demand. Nanotechnology has an important role nowadays in creating new antimicrobial agents through increasing the penetration power, shape and
increasing the surface area. In the present study, STNT, STNS, HTNT and HTNS showed good antibacterial activity at different concentrations against Gram Positive and Gram-negative bacteria besides antifungal activity with increasing antimicrobial activity towards the STNT shape than other shape. The synthesized nanomaterials possessed a well-developed surface chemistry; chemical stability and the shape of the nanomaterials are the main factors responsible for the antimicrobial activity.

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