Comparison of antibacterial and antifungal activities of 5-amino-2-mercaptobenzimidazole and functionalized NiO nanoparticles

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Abstract

5-Amino-2-mercaptobenzimidazole (AMB), non-functionalized nickel oxide (n-NiO), and functionalized nickel oxide (f-NiO) nanoparticles were studied for antibacterial and antifungal activities, where the functionalization of nickel oxide nanoparticles was carried out by AMB. The particle sizes of synthesized n-NiO and f-NiO nanoparticles were measured to be 16.07 and 20.86 nm, respectively. The XRD results of n-NiO and f-NiO nanoparticles were in perfect match with the diffraction pattern of NiO published in the JCPDS File No. 89-5881, which indicates that there is no effect on the crystal structure due to functionalization. FT-IR spectral studies show that f-NiO nanoparticles effectively bind with AMB by azomethine nitrogen. Furthermore, HR-SEM and EDAX results confirm the surface morphology and functionalization, respectively. The antimicrobial activity of AMB, n-NiO, and f-NiO nanoparticles dispersed in water was investigated. The antibacterial activity was evaluated against the bacteria Staphylococcus aureus and Pseudomonas aeruginosa, while the antifungal activity was evaluated against the fungi Aspergillus niger by using the agar well-diffusion method. The f-NiO nanoparticles exhibit excellent antibacterial and antifungal activities compared with n-NiO nanoparticles and AMB. The increased effect of f-NiO nanoparticles might be due to enhanced dispersibility and interaction of NiO nanoparticles with membrane and intracellular proteins of bacteria and fungi.

Keywords: Nickel oxide; Benzimidazole; Antibacterial activity; Antifungal activity

1. Introduction

The engineered inorganic nanoparticles display unique physical and chemical properties, which can be used in numerous physical, biological, biomedical, and pharmaceutical applications [1,2]. Recent research achievements offer the possibility of generating new types of surface-coated nanoparticles for enhanced applications [3]. The current interest in the development of new antimicrobial agents can be partially ascribed to the increasing emergence of bacterial
resistance against antibiotic therapy and to newly emerging pathogens [4]. Many problems remain unsolved for most available antimicrobial drugs, in spite of advancement in antibacterial therapy. Benzimidazole derivatives are of wide interest because of their diverse biological activity and clinical applications. The ring system of benzimidazole was present in numerous antiparasitic, fungicidal, antioxidant, and anti-inflammatory drugs [5,6]. However, the general antimicrobial activity of benzimidazole derivatives has not been extensively investigated. Zhang et al. studied about the photogeneration of reactive oxygen species on uncoated silver, gold, nickel, and silicon nanoparticles to estimate their antibacterial efficacy. The research findings show that nickel possesses excellent antimicrobial effect compared with other metal nanoparticles in the same condition. Another study shows that nickel nanoparticles possess excellent antibacterial activity against E. coli, Lactobacillus casei, Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis [7]. In this investigation, we report the synthesis of highly stable nickel oxide (NiO) nanoparticles and nickel oxide nanoparticles surface functionalized with 5-amino-2-mercaptobenzimidazole (AMB) to study the influence on antibacterial and antifungal effects. Efforts were made to understand the underlying molecular mechanism of such antimicrobial actions. The effect of the nanoparticles was found to be significantly more pronounced on the gram-negative strains, irrespective of whether the strains are resistant or not, than on the gram-positive organisms. For discussion, non-functionalized nickel oxide nanoparticles are referred to as n-NiO, functionalized nickel oxide nanoparticles as f-NiO and 5-amino-2-mercaptobenzimidazole as AMB.

2. Materials and methods

2.1. Raw materials

The nickel oxide (NiO) nanoparticles were synthesized using nickel nitrate (Sigma–Aldrich) and sodium hydroxide pellets (Merck). The nickel oxide nanoparticles were surface functionalized with 5-amino-2-mercaptopbenzimidazole (Sigma–Aldrich). All the chemicals were of analytical grade and used without further purification.

2.2. Synthesis of NiO nanoparticles

The NiO nanoparticles were synthesized by chemical precipitation method. Nickel nitrate (Ni(NO₃)₂·6H₂O) and sodium hydroxide (NaOH) were taken in 1:4 molar ratios and dissolved completely in deionized water separately. The salt solution was stirred well using a magnetic stirrer and sodium hydroxide solution was added in drops to obtain a pale green precipitate. The solution was continuously stirred to obtain a homogeneous medium. Furthermore, the precipitated solution was ultrasonicated for 1 h to obtain fine particle sizes. Later, the precipitate was washed several times with deionized water and annealed at 400 °C for 4 h to remove moisture from nickel hydroxide to obtain nickel monoxide (NiO) nanoparticle.

2.3. Surface functionalization of NiO nanoparticles

The surface functionalization of NiO nanoparticles was carried out by mixing 2.0 g of NiO nanoparticles, 2 g of AMB, and 40 ml ethanol in a beaker. The solution was stirred vigorously using magnetic stirrer for 3 h. Then, the amine-functionalized NiO nanoparticles were collected by filtration using Whatman filter paper (No. 1) and rinsed with acetone; later the sample was dried under vacuum for 12 h.

2.4. Test microorganism and maintenance

Test pathogenic bacteria, such as P. aeruginosa, S. aureus, and fungi Aspergillus niger, were used for in vitro antimicrobial activity. These selected pathogenic strains were obtained from microbiological division (Jayagen Biologics Analytical Laboratory, Chennai). The pathogenic bacteria was maintained on nutrient agar slants and stored at 4 °C with regular transfers at monthly intervals. For long preservation, 25% glycerol nitrate agar was added to the slants.

2.5. Measurements

The synthesized n-NiO and f-NiO nanoparticles were investigated by X-ray diffraction analysis (XRD-XPert Pro Philips) for structural confirmation and particle sizes were calculated using Debye Scherrer’s formula. The functionalization of AMB on nickel oxide nanoparticles was analyzed by FT-IR spectroscopy (Nicolet Magna 550 FT-IR spectrometer) and confirmed with EDAX results. The morphological changes were evaluated using HR-SEM (Modelno.S3400, Hitachi).

2.6. Liquid suspension

The liquid suspensions of the samples AMB, n-NiO and f-NiO nanoparticles were prepared by dispersing
them in deionized water in separate containers. For antimicrobial assay, a micropipette was used to measure the various concentrations of 20, 40, 60, 80, and 100 µl from the prepared liquid suspensions of samples.

2.7. Agar well-diffusion antibacterial activity

The biological culture was performed using an agar well-diffusion method. Antimicrobial activities of AMB, n-NiO, and f-NiO nanoparticles were evaluated against both gram-negative (P. aeruginosa) and gram-positive (S. aureus) bacteria. The antibacterial activity was evaluated by using the modified Kirby–Bauer disk-diffusion method. In brief, the pure cultures of organisms were subcultured in Müller–Hinton broth at 35 ± 2 °C on a rotary shaker at 160 rpm. For bacterial growth, a lawn of culture was prepared by spreading the 100 µl fresh culture having 10⁶ colony-forming units (CFU)/ml of each test organism on nutrient agar plates with the help of a sterile L-rod spreader. Plates were left standing for 10 min to let the culture get absorbed. Then, 8-mm wells were punched into the nutrient agar plates for testing the activity of antimicrobial nanoparticles. Wells were sealed with one drop of molten agar (0.8% agar) to prevent leakage of nanoparticles from the bottom of the wells. Using a micropipette, 100 µl (50 µg) of nanoparticles suspension was poured onto each of five wells on all plates. After overnight incubation at 35 ± 2 °C, the different levels of zone of inhibition were measured. Non-functionalized NiO nanoparticles and antibiotic tetracycline were used as control.

2.8. Agar well-diffusion antifungal activity

For the antifungal assay, the ready-made potato dextrose agar medium (Himedia, 39 g, Mumbai, India) was suspended in distilled water (100 ml) and heated until it dissolved completely. The medium and Petri dishes were autoclaved at a pressure of 15 Pascal (Pa) for 20 min. The medium was poured into sterile Petri dishes under aseptic conditions in a laminar flow chamber. When the medium in the plates solidified, a fungal disk of A. niger was placed at the center of Petri dish (90 mm diameter) between the test materials. For agar inoculation, wells were scooped out with 8 mm sterile cork borer and the lids of the dishes were replaced. To each well, different concentrations of test solution were added. Control was maintained with n-NiO nanoparticles and amphotericin B. The distance from fungal disk to the edge of the specimens was fixed at 15 mm. The fungi were then incubated at 30 °C for 7 days.

3. Results and discussion

3.1. Structural analysis

The powder XRD pattern of n-NiO and f-NiO nanoparticles is shown in Fig. 1. It reveals that the XRD peaks of n-NiO and f-NiO samples are in a perfect match with the diffraction pattern of NiO published in the JCPDS File No. 89-5881 [8]. The XRD peaks were indexed using XPowder software packages. The diffraction peaks of the as-prepared NiO nanoparticles were at 2θ = 37.13, 43.22, 62.77, 75.20 and 79.19° corresponding to the Miller indices or lattice planes of (111), (200), (220), (311) and (222), respectively, which can be indexed to the face-centered cubic structure of NiO nanoparticles. The XRD pattern of NiO nanoparticles shows broad peaks, which confirms the formation of small-sized nanoparticles. The particle sizes of nanoparticles was determined using the Debye–Scherrer’s relation d = (0.9*λ)/(βcosθ), where β is the full width at half maximum in radians, λ is the wavelength of X-rays used, and θ is the Bragg’s angle [9]. The particle sizes were calculated for the most prominent peaks, and the average particle sizes of the n-NiO and f-NiO nanoparticles were found to be around 16.07 and 20.86 nm, respectively, as shown in Table 1. The functionalization of nickel oxide nanoparticles with AMB does not affect the polycrystalline nature of the sample. The effective binding of the AMB on NiO nanoparticles were evidenced from peaks obtained at 20 values of f-NiO between 10 and 30°, which was absent in n-NiO. The particle sizes of n-NiO and f-NiO samples calculated from the most...
The intense peak of $2\theta$ 37.13, 43.22, and 62.77° were matched well with each other, whereas a slight deviation exists when calculated with the $2\theta$ values 75.20 and 79.19°. The d-spacing values and the (hkl) values were same for both the samples.

### 3.2. HR-SEM and EDAX analysis

Fig. 2a and b shows the HR-SEM image and EDAX spectrum of n-NiO nanoparticles, respectively. Similarly, the HR-SEM image and EDAX spectrum of f-NiO nanoparticles are shown in Fig. 3a, b. The surface morphology of n-NiO and f-NiO nanoparticles was uniform, and the particle sizes were within the nanometer range as obtained from Debye Scherrer's calculation. The particle size of f-NiO nanoparticles slightly increases in comparison with n-NiO nanoparticles. The EDAX spectrum results of n-NiO and f-NiO nanoparticles provide the evidence of functionalization. The presence of carbon, sulfur, and nitrogen in f-NiO nanoparticles is the proof for surface functionalization of AMB on NiO nanoparticles, which was absent in n-NiO nanoparticles.

### 3.3. FT-IR spectral studies

Fig. 4 shows the FT-IR spectra of n-NiO, f-NiO, and AMB. In the FT-IR spectra of n-NiO, the broad band centered at 3426 cm$^{-1}$ was attributed to OH stretching of hydrogen-bonded water on the surface of nanoparticles and the band at 1628 cm$^{-1}$ is assigned to H–O–H bending vibrations mode presented due to the adsorption of water in air during the preparation of FT-IR sample disks in an open air atmosphere. The sharp band at 1383 cm$^{-1}$ is attributed to the O–C=O symmetric and asymmetric stretching vibrations. The

### Table 1

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<th>Particle sizes calculated using Debye Scherrer’s formula for various prominent peaks of n-NiO and f-NiO nanoparticles.</th>
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<tr>
<td>n-NiO nanoparticles</td>
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<td>$2\theta$</td>
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<tr>
<td>37.57</td>
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<td>43.17</td>
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<td>62.74</td>
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<td>75.20</td>
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<td>79.19</td>
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<td>Average particle size</td>
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Fig. 2. (a) HR-SEM image of n-NiO nanoparticles; (b) EDAX spectrum of n-NiO nanoparticles.
moderate peak at 1018 cm$^{-1}$ may be due to single C–O bond stretching mode. The bands at 529 and 423 cm$^{-1}$ correspond to δ(OH) and υ(NiO) vibrations, respectively. The sharp peak at 3363 cm$^{-1}$ and broad peak at 3189 cm$^{-1}$ in neat AMB and f-NiO nanoparticles attributed to N–H (amine) and C–H (aromatic) stretching vibration [10]. The presence of band at 3362 cm$^{-1}$ and 2656 cm$^{-1}$, which corresponds to stretching vibration of υ(N–H) and υ(S–H), respectively, indicates the coordination of NiO nanoparticles to the AMB does not take place through sulfur and nitrogen atom via deprotonation. The characteristic band at 2953 cm$^{-1}$ and 2870 cm$^{-1}$ in neat AMB and f-NiO nanoparticles is attributed to C–H (aliphatic) stretching. The neat AMB and f-NiO exhibited a medium-intensity band at 2580 cm$^{-1}$ that was assigned to stretching vibration of S–H group, which indicates the thiol form of the ligand. The bands at 1635 cm$^{-1}$ and 1615 cm$^{-1}$ attributed to υ (–C==N) stretching vibration of AMB and f-NiO, whereas –C==C aromatic stretching vibration bands were observed at 1526, 1493, and 1467 cm$^{-1}$. The bands at 1370–1400 cm$^{-1}$ were assigned to –S–C stretching vibration of mercapto group. The υ(C–O) phenolic stretching band observed at 1274 and 1223 cm$^{-1}$ in both AMB and f-NiO nanoparticles indicates that the bonding is not through phenolic oxygen. The doublet at 1197–1170 cm$^{-1}$ and 1132–1117 cm$^{-1}$ in AMB and f-NiO was assigned to O=S=O group. A weak band appearing at 1038 cm$^{-1}$ in the spectrum of f-NiO nanoparticles was assigned to –OCH$_3$ group but the same was absent in AMB. The coordination of the water molecules to metal ions was further supported by the appearance of bands in the region 853–871 cm$^{-1}$ attributed to rocking and wagging mode of water. The bands at 600–632 and 680–756 cm$^{-1}$ correspond to the –C–S–H stretching of the mercapto group, which was found in both AMB and f-NiO. The bands at 529 and 424 cm$^{-1}$ in f-NiO correspond to δ(OH) and υ(NiO) vibrations of NiO nanoparticles [11,12]. The FT-IR spectra of AMB, n-NiO, and f-NiO reveal that the NiO nanoparticles effectively bind with AMB through azomethine nitrogen but it was a weak physical bonding. There was no evidence for strong chemical bonding of NiO nanoparticles with AMB.
3.4. Antibacterial activity

AMB, n-NiO, and f-NiO nanoparticles were tested for their in vitro antibacterial activity against gram-positive bacteria *S. aureus* and gram-negative bacteria *P. aeruginosa* at 20–100 μg/ml concentration (Figs. 5 and 6). The in vitro antimicrobial activity of the tested compounds was assessed by minimum inhibitory concentration (MIC) using the broth-dilution method (National Committee for Clinical Laboratory Standards, 1982). Tetracycline (100 μg/ml) was used as control for comparison, and the data are presented in Table 2. The results of antibacterial screening (Table 2) reveal that surface-functionalized nanoparticles displayed increased activity compared with n-NiO nanoparticles. A recent study shows that extracellular nickel
ions cannot pass easily through the ion channels of the cell membrane, but small nickel particles can be uptaken by cells and then release Ni\textsuperscript{2+} intracellularly. So nickel in the form of nanoparticles releases nickel ions more effectively and has high bactericidal activity due to its higher surface-area-to-volume ratio. The results show that f-NiO nanoparticles and AMB exhibit a marked degree of activity against gram-negative bacteria \textit{P. aeruginosa} at the minimum inhibitory concentration (MIC) of 20 \textmu g/ml, whereas n-NiO nanoparticles exhibit antibacterial effect at MIC of 80 \textmu g/ml. The MIC of n-NiO and f-NiO nanoparticles against gram-positive bacteria \textit{S. aureus} was found to be 60 \textmu g/ml. However, there exists an enhanced antibacterial activity in f-NiO nanoparticles sample, with 12 and 15 mm zone formation at 80 and 100 \textmu g/ml concentration, respectively, whereas in n-NiO nanoparticles it reads 6 mm for both 80 and 100 \textmu g/ml concentration. The AMB does not exhibit antibacterial behavior for concentration 20–100 \textmu g/ml against gram-positive bacteria \textit{S. aureus} but possesses excellent antibacterial effect even at lower concentration of 20 \textmu g/ml against gram-negative bacteria \textit{P. aeruginosa}. In general, the activities of antibacterial agent will be better against gram-negative bacteria than gram-positive bacteria due to the multilayered thick peptidoglycan cell wall of gram-positive bacteria, whereas in gram-negative bacteria the cell wall comprises a thin, single layer of peptidoglycan. The NiO nanoparticles exhibit high antimicrobial activities; however, its dispersibility in the aqueous culture medium is lower than other metal oxide nanoparticles. The small particle size of NiO nanoparticles and surface functionalization could enhance its dispersibility, and also the presence of extracellular Ni\textsuperscript{2+} could interfere with the intracellular Ca\textsuperscript{2+} metabolism and cause cellular damage. Furthermore, the small particle size of NiO nanoparticles made them easier to penetrate into bacterial cell membrane. Heavy metals have a greater affinity toward protein molecules, which binds them to functional groups of proteins, resulting in protein denaturation and causing bacterial cell death [13]. In f-NiO nanoparticles, the combined effect of both nickel oxide nanoparticles and AMB was more effective on bacteria. The AMB has both electron-donating and electron-withdrawing groups as substitutions on the benzene ring, which attribute to excellent antibacterial property [14]. The synergistic effect of both NiO nanoparticles and AMB was responsible for enhanced antibacterial effect. Furthermore, the role of oxidative stress from the generation of \textsuperscript{1}O\textsubscript{2} was also evident on NiO nanoparticles due to surface functionalization.

3.5. Antifungal activity

AMB, n-NiO and f-NiO nanoparticles were also evaluated for their antifungal activity against \textit{A. niger} by using the agar well-diffusion method (Margery Lindsay, 1962), using amphotericin B as control (Fig. 7). The results are summarized in Table 3. The antifungal activity results (Table 3) indicate that all the

![Fig. 7. Antifungal effect by Agar Well cut diffusion method against \textit{Aspergillus niger} of a) AMB; b) n-NiO nanoparticles; c) f-NiO nanoparticles.](image)

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<th>S. No.</th>
<th>Sample</th>
<th>Aspergillus niger (in mM)</th>
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<tr>
<td></td>
<td>AMB</td>
<td>--</td>
</tr>
<tr>
<td>1</td>
<td>n-NiO</td>
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<tr>
<td>2</td>
<td>f-NiO</td>
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Table 3: Zone of inhibition for antifungal effect of AMB ligand, n-NiO and f-NiO nanoparticles by Agar Well cut diffusion method.
examined samples have similar effects with MIC value of 80 μg/ml; however, f-NiO nanoparticles show enhanced toxicity compared with n-NiO nanoparticles, with zone inhibition value of 16 mm. The nanoparticles penetrate easily into the fungal cell membrane due to their small particle size, bind to functional groups of proteins, and phosphorous- and sulfur-containing compounds such as DNA, and cause fungal cell death \[15\]. Compounds possessing 2-hydroxy 4-methyl and 4-nitro substitutions on the phenyl ring show remarkable growth inhibition, which may be the main reason for the antifungal effect of AMB. The combined effect of AMB and nickel oxide nanoparticles was less pronounced in the antifungal effect of f-NiO nanoparticles compared with antibacterial effect.

4. Conclusions

The surface functionalization of nickel oxide nanoparticles with 5-Amino-2-mercaptobenzimidazole (AMB) was expected to show some increased antimicrobial activities compared with non-functionalized nanoparticles. The particle sizes of synthesized n-NiO and f-NiO nanoparticles measured 16.07 and 20.86 nm, respectively. The FT-IR analysis shows several bands, for example, at 3363 cm\(^{-1}\) corresponding to N–H (amine), 1635 and 1615 cm\(^{-1}\) attributed to v (–C≡N) stretching vibration, and 600–632 and 680–756 cm\(^{-1}\) corresponding to –C–S–H stretching of the mercapto group, which confirms the effective binding of AMB on nickel oxide nanoparticles. Furthermore, HR-SEM results explain the surface morphology, and EDAX shows the presence of sulfur, nitrogen, and carbon as an evidence of AMB binding on nickel oxide nanoparticles. The f-NiO nanoparticles show enhanced antibacterial and antifungal activities compared with the unmodified n-NiO nanoparticles. The nickel oxide nanoparticles penetrate the bacterial cell membrane and bind to the functional groups of protein, leading to protein denaturation. In addition to the effect of nickel oxide nanoparticles, the f-NiO nanoparticles possess AMB on its surface, which was the reason for enhanced antimicrobial behavior. As the AMB possesses both electron-donating and electron-withdrawing groups as substitutions on the benzene ring, it exhibits excellent antibacterial property. The synergistic effect of both NiO nanoparticles and AMB was responsible for enhanced antimicrobial effect.

References