

Antifungal activities of Zerovalent Cobalt and Nickel nanocomposites Integrated with Crosslinked Chitosan-Humic acid against some *Candida species*

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Abstract

Zerovalent Cobalt and Nickel nanocomposites with crosslinked Chitosan-Humic acid were synthesized. Humic acid was used for intramolecular cross-linking of the chitosan linear chains to increase the active sites on the chitosan biopolymer which was further used as stabilizer to synthesize zerovalent metal nanoparticles by the reduction of metal chlorides with sodium borohydride. Characterization of the products was carried out using Infrared spectrophotometer, Scanning Electron Microscope (SEM), Transmission Electron Microscope (TEM) and Powder X-Ray Diffractometer (PXRD). Chitosan, humic acid and the nanocomposites were investigated for antifungal activity against four *Candida species* (*C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsilosis*). Significant antifungal activity was observed with the highest against *Candida albicans*.

Keywords: Chitosan, Humic acid, Antifungal property, Metal nanoparticle

1 Introduction

There is an increasing need to develop novel agents to treat fungal infections that have become unresponsive to standard antimicrobial therapy [1]. *Candida* species are the most common cause of yeast fungal infections worldwide with *Candida albicans* (*C. albicans*) being the most commonly isolated species. Some strains of *Candida*, example *Candida auris*, are often resistant to multiple antifungal drugs [2]. Chitosan (CTS), a natural, abundant and non-toxic polymer has been reported to exhibit antimicrobial activity and as such has been applied in many fields, such as agriculture, biotechnology, cosmetics, medical, textiles, wastewater treatment etc [3-4]. Its antifungal activities has been studied against various fungi species [5-7]. The antifungal activities of chitosan and various modified chitosans were

compared in relation to *Saprolegnia parasitica* (*S. parasitica*) growth in vitro, in which methylpyrrolidinone chitosan, N-carboxymethyl chitosan and N-phosphonomethyl chitosan exerted effective fungistatic action against *S. parasitica* [5]. Due to its reactivity, chitosan can be modified easily to produce new derivatives [8-12]. Metal complexes of chitosan have also been shown to have enhanced antimicrobial activity due to the presence of the metals [4].

Humic acid(HMA) also exhibits antimicrobial properties which increases in the presence of metals with which they form chelates due to its numerous binding sites. The antifungal activity of humic acid has been studied against some plant fungi spp. with about 30-50% inhibition rate [13-14].

In this work, the synthesis, characterization, and antifungal activities of crosslinked chitosan-humic acid with zerovalent

metals ZnMe-CTS-HMA are described against four *Candida spp.*

Our group has previously reported the preparation and characterisation of crosslinked chitosan with 2,6-pyridinedicarboxylic [15] and humic acid [16] with zerovalent metals for environmental remediation. The condensation reaction occurs between the carboxylic groups present in acids and amino groups of CTS in the presence of carbodiimide.

2 Materials and Method

2.1 Materials

Chitosan with 80% degree of deacetylation was prepared from chitin which was obtained from snail shells as previously described [15]. Sodium borohydride (NaBH_4), humic acid and N,N'-Carbonyldiimidazole (CDI) were purchased from Sigma Aldrich, United State. Other reagents like cobalt chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), nickel chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$), sodium hydroxide, hydrochloric acid, acetone, acetic acid and NaNO_3 were obtained from the stock at Department of Chemistry, Federal University of Agriculture, Abeokuta. All aqueous solutions were made with ultrahigh purity water purified with an ultrapure water system Milli-Q Plus (Millipore Co). The *Candida* strains used include *C. albicans* (ATCC 28516) *C. glabrata* (ATCC 15126) *C. tropicalis* (ATCC 750) and *C. parapsilosis* (ATCC 22019) obtained from the University of Ibadan Teaching hospital, Ibadan. Sabouraud dextrose agar was obtained from the stock at the Microbiology department, Federal University of Agriculture, Abeokuta and prepared as described by standard methods [17].

2.2 Characterisation

Infrared spectra were recorded on a Shimadzu Fourier Transform Infra-red (FT-IR-8400S) spectrophotometer. Scanning electron microscope (SEM) images were obtained using a JEOL JSM 840 scanning electron microscope. Transmission electron microscopy (TEM) images were obtained using a ZEISS LIBRA® JEOL JEM 1210 microscope at 100 kV acceleration voltage. X-ray powder diffraction

(XRD) patterns were recorded on a Bruker D8 Discover equipped with a Lynx eye detector, using $\text{Cu-K}\alpha$ radiation (1.5405 Å, nickel filter). Data were collected in the range from $2\theta = 5^\circ - 100^\circ$, scanning at 1°min^{-1} with a filter time-constant of 2.5 s per step and a slit width of 6.0 mm. Samples were placed on a zero background silicon wafer slide. The X-ray diffraction data were treated using Eva (evaluation curve fitting) software. Baseline correction was performed on each diffraction pattern

2.3 Synthesis of Cross-Linked Chitosan-Humic acid(CTS-HMA)and Zerovalent metal Nanocomposite

The crosslinked chitosan with humic acid was prepared as previously described (16) and the product CTS-HUM (Figure 1) also obtained by freeze-drying. To a well stirred ethanolic solution of metal salt ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ -12.10 g, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ -12.15 g) was added CTS-HMA (3.0 g). 1.0 M NaBH_4 solution was then added drop-wisely to the Me-CTS-HMA mixture with continuous stirring of the resulting solution [4, 15,16]. Both reactions resulted in the formation of black coloured particles which were separated from the liquid phase using vacuum filtration. The solid particles were washed at least three times with 25 mL portions of absolute ethanol to remove all of the water and products collected by freeze drying.

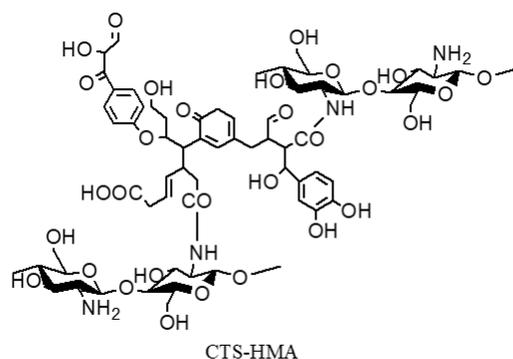


Figure 1: Crosslinked Chitosan-Humic acid

2.4 Antifungal assay

The antifungal assay was carried out on the CTS- HMA, ZnCo(CTS- HMA), ZnNi(CTS-

HMA), the free chitosan and humic acid against the four *Candida* strains, using the agar well diffusion method [4, 17]. Stock solutions of the products were prepared by dissolving 0.1 g in 3mL sterilized water. Holes (6.00 mm) were drilled in Sabouraud dextrose agar inoculated with the test organisms in petri dishes. 10, 20 and 40 mg of the samples respectively were dispensed in the sterile paper discs (Whatman: 1.6 mm) with absorbed spice extract (30 μ L/disc) placed on the agar at certain intervals by pressing gently. After the plates were incubated at $35\pm 0.1^\circ\text{C}$ for 48 h, zones of inhibition were measured in mm and taken as a measure of the inhibitory power of the samples against the particular test organism. The experiments were repeated in duplicate for all of the *Candida* test strains.

3 Results and Discussions

3.1 Synthesis and Characterisation

The zerovalent metal humic acid – chitosan nanocomposites was obtained as a powder and therefore could be dispersed into aqueous solutions. The observed colour change to black during the reaction was due to the reduction of the metal ions to their zerovalent state [15].

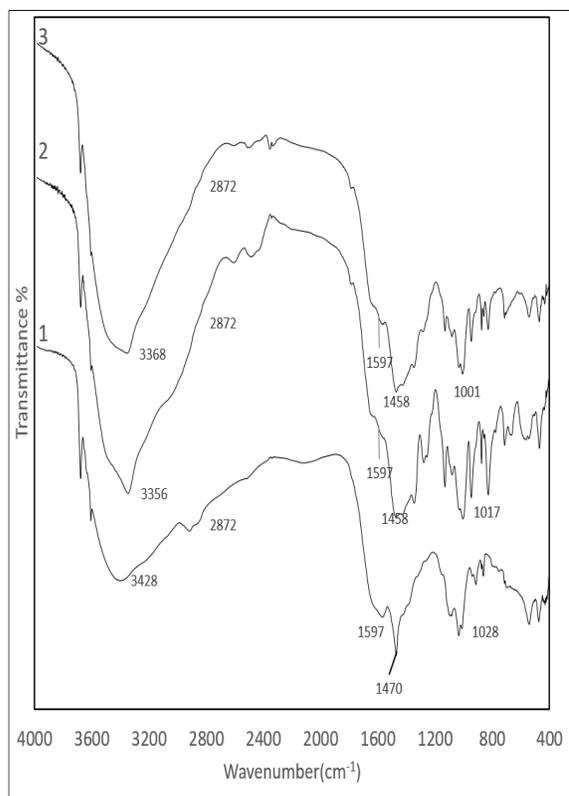


Figure 2: FT-IR Spectra: 1-CTS-HMA; 2-ZvCo-CTS-HMA; 3- ZvNi-CTS-HMA

The infrared spectra of the crosslinked chitosan-humic acid and its iron nanocomposites have been previously discussed [16]. The IR spectra are as shown in Figure 2. The broad peak at around $3400\text{--}3300\text{ cm}^{-1}$ assigned to --NH_2 and --OH vibration stretch showed a maximum peak at around 3400 cm^{-1} for CTS-HMA. The peak becomes sharper and has a peak maximum at a lower frequency (3300 cm^{-1}) for ZvCo(CTS-HMA) and ZvNi(CTS-HMA) due to changes in the --NH and --OH environment in the presence of the metal nanoparticles.

The amine groups of chitosan reacts with the carboxylic groups of humic acid electrostatically for formation of CTS-HMA (16). A sharp peak around 1470 cm^{-1} observed in CTS-HMA broadens up to 1300 cm^{-1} in the spectra of the nanocomposites.

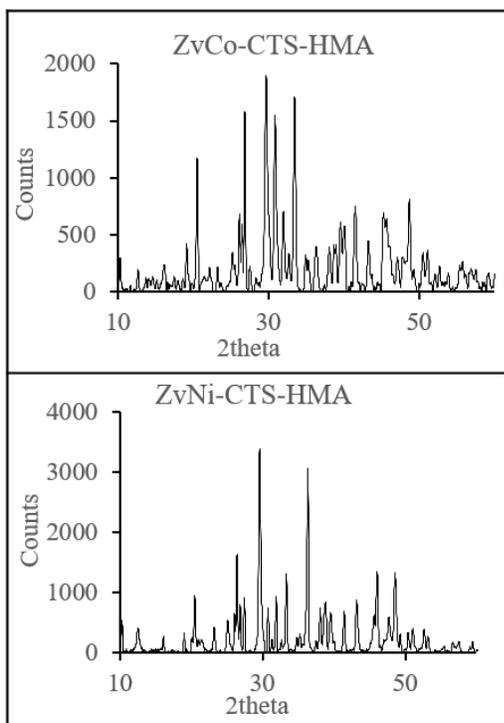


Figure 3: X-ray diffractograms of ZnCo-CTS-HMA and ZnNi-CTS-HMA.

The XRD data for the metal nanocomposites are presented in Figure 3. In agreement with literature, the characteristic peak at 20θ for crystalline CTS is not present in the ZnMe-CTS-HMA compounds. The peaks observed here are sharper and more intense showing the metal nanocomposites are more crystalline and less amorphous than the organic starting material CTS-HMA which is attributed to the presence of the metals [12,16].

Figure 4 shows the scanning electron micrographs of ZnCo-CTS-HMA and ZnNi-CTS-HMA. The images displayed significant morphological changes when compared with image of the CTS-HMA which is attributed to electrostatic interactions between the crosslinked CTS-HMA and the zerovalent metal [16]. The micrograph of the metal nanocomposite looked more porous and much less smoother than that of the previously described image of CTS-HMA [16]. CTS has been reported to lose its smooth surface on crosslinking and with metal complexation. This is in agreement with previous works on modified chitosan (8,18).

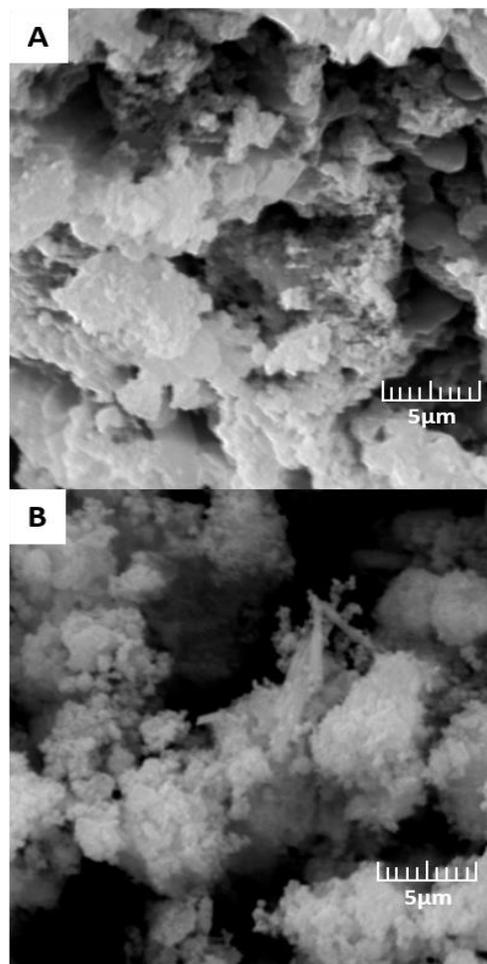


Figure 4: SEM Diagrams: A-ZnCo-CTS-HMA; B - ZnNi-CTS-HMA

The TEM diagram (Figure 5) showed the dispersed metal nanoparticles (with sizes $<50\text{nm}$) encapsulated in the crosslinked CTS-HMA matrix. The zerovalent metal are protected by the CTS-HMA system and oxidation prevented [15].

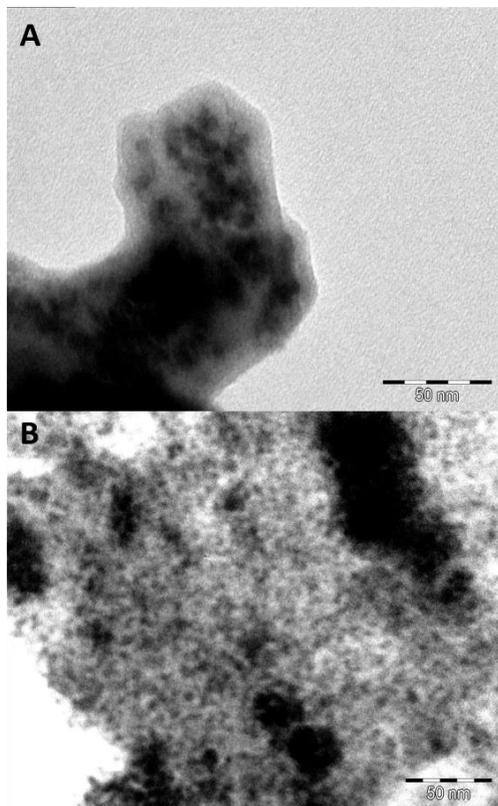


Figure 5: TEM diagram A-ZvCo-CTS-HMA B- ZnNi-CTS-HMA.

3.2 Antifungal activities

The result of the antifungal test is summarized in Figures 6 and 7. The antifungal activity of the test solutions increased with increasing concentrations from 10 – 40 mg/mL against all the test organisms (Figure 6). The test solutions were most active against *C. albicans* with ZnNi-CTS-HMA being most active with more than 50% zone of inhibition (Figure 7). HMA showed a strong activity against *C. albicans* and *C. glabrata* even at a low concentration (10 mg/mL) [14]. On the average, chitosan showed a better activity than CTS-HMA at all concentrations. The CTS-HMA activity was greatly improved when combined with the metals nanoparticles especially against *C. albicans* and *C. tropicalis* [12]. The ligands and the metal nanocomposites all displayed low activity against *C. tropicalis* in comparison to *C. albicans*.

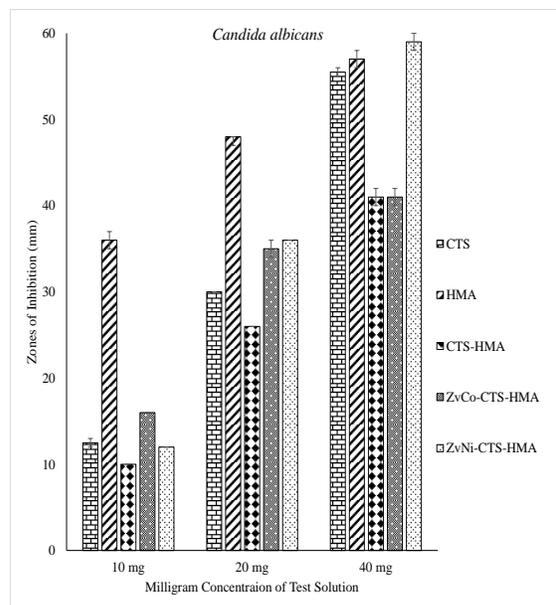


Figure 6: Mean zone of inhibition (mm) of 10, 20 and 40 mg/mL of the test solutions against *Candida albicans*.

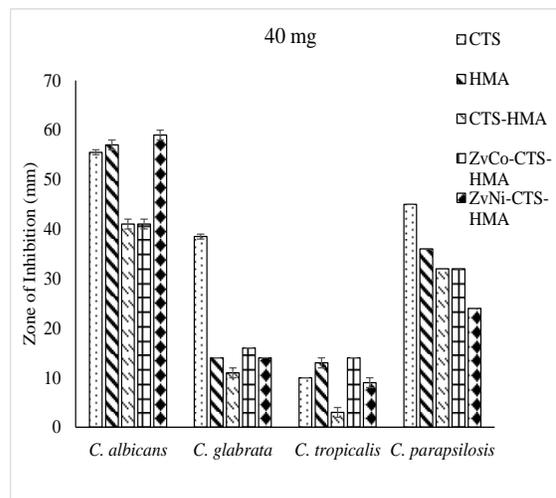


Figure 7: Mean zone of inhibition (mm) of 40 mg/mL of the test solutions on the *Candida* species.

4 Conclusions

We synthesized a nontoxic organic-inorganic polymeric metal nanocomposite from the reaction of crosslinked chitosan-humic acid, with cobalt and nickel salts. From the TEM figures, it showed that the metal nanoparticles

were formed and well encapsulated in the CTS-HMA matrix. The products all showed antifungal properties against the *Candida* species at different degrees especially against *Candida albicans*. Chitosan showed stronger activities against the species than CTS-HMA, however the presence of the metal nanoparticles, enhanced the antifungal properties of the CTS-HMA ligand.

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