Efficiency of Hydrothermal Synthesis of Nano/Microsized Copper and Study on In Vitro Antifungal Activity

AHLAM NEMATI1,2, SASAN SHADPOUR1,2, HAMID KHALAFBEYGI1, SIMIN ASHRAF3, MOHAMMAD BARKHI2,4, AND MOHAMMAD REZA SOUDI3

1Department of Chemistry, Islamic Azad University, Karaj, Iran
2Nano System Research Team (NRTeam, NGO), Karaj, Iran
3Department of Biology, Al-zahra University, Tehran, Iran
4Department of Chemistry, Applied-Scientific University, Karaj, Iran

In this study, the synthesis and characterization of copper nano/microparticles in the presence of (2,2',2''-(ethane-1,2-diylbis(azanetriyl))tetraacetohydrazide) as a capping and reducing agent under hydrothermal conditions for use in biological conditions were investigated. The effects of reductant ligand/copper ion, concentration ratios, reaction temperatures, and reaction time on the various morphologies of copper nano/microparticles were studied. The obtained particles have been characterized by X-ray diffraction, scanning electron microscopy, energy-dispersive X-ray spectrum analysis (EDAX), and zeta potential analysis. Further, the formation of particles with different morphologies was confirmed. The biological and antifungal effects of these selected particles were compared with those of the Bordeaux mixture. Experiments were conducted on Alternaria alternata, Alternaria solani, and Fusarium expansum as three types of phytopathogenic fungi and Penicillium as a non-phytopathogenic fungus. The results obtained showed that not only were the nano/microparticles more effective than Bordeaux mixture in killing phytopathogenic fungi, but also these particles did not have a fungicidal effect on the non-phytopathogenic fungus, Penicillium, which is an advantage of the obtained nano/microparticles.

Keywords Antifungal; Electron microscopy; Hydrothermal; Morphology; Nanoparticles.
INTRODUCTION

Due to their unique characteristics, the synthesis of metallic nanoparticles (NPs) enjoys an increasing popularity. NPs have various applications in fields as varied as medicine and biotechnology [1–3], catalysis [4, 5], imaging [6–7], and data storage [8]. Silver and copper NPs, in particular, have a larger specific surface area and have traditionally been well-known as antimicrobial agents [9–11]. Today, through the design of new NPs and the study of their surface morphologies, we can take advantage of their antifungal activity, which mitigates or even prevents fungal colonization, for medical purposes. Several antifungal agents have been recently proposed [12–14]. The fungicidal effects of copper compounds have been known for many years, particularly since the important discovery of “Bordeaux mixture” in 1887 [15]. The mixture is composed of copper sulfate and calcium hydroxide and is an effective non-systemic fungicide capable of controlling fungal diseases caused by Plasmopara viticola, Phytophthora spp., Diplodia, Venturia inaequalis, Septoria, and natalensis, among others [16]. It should be noted that there have been few studies on the applicability of various forms of copper ions such as CuO and Cu2O to controlling plant diseases [17]. In this study, a new method for the synthesis of copper NPs was employed using 2,2',2''''-(ethane-1,2-diyldis(azanetriyl)) tetraacetoxyhydrozide (EDDH), which can act as both a reducing and a capping agent. The antifungal effects of these NPs on the fungal species Alternaria alternata, Alternaria solani, Fusarium oxysporium, and a Penicillium sp. were compared with those of the Bordeaux mixture in vitro, showing the inhibition of colony formation after Petri dishes containing these fungi were sprayed with water-based suspensions of these particles and the stock solution of Bordeaux mixture. The results showed that the copper NPs are far more effective than Bordeaux mixture in killing phytopathogenic fungi.

MATERIALS AND METHODS

All the chemicals used were of analysis grade, purchased from commercial sources, and were, hence, used without any further purification. Deionized water was used in all the experiments. The ligand EDDH was synthesized according to the literature method patented as EP 1808428 (A1) [18].

Scanning electron microscopy (SEM) images and EDAX analyses were obtained on a Philips XL-30ESEM, equipped with an X-ray energy-dispersive detector, and were recorded by Philips XL30 TMP (F.E.I. Company, Hillsboro, OR, USA). The Sputter Coater (BAL-TEC SCD 005 Balzers, Switzerland) was used for gold coating. X-ray diffraction (XRD) patterns of the samples were recorded on EQUINOX 3000 (Stratham, NH, USA) X-ray powder diffractometer with Cu Kα (λ = 1.5406 Å) radiation. The UV-Vis spectroscopy measurements of copper NPs were performed with a Varian spectrophotometer (model Cary 100, Australia). Zeta potential was measured using the laser zeta meter (Malvern zeta seizer 2000).

Copper NPs were synthesized by the hydrothermal reaction of EDDH and the metal salt copper acetate in a stainless steel autoclave at an autogenous pressure. In this study, 50 mg (0.25 mmol) of Cu(CH3CO2)2.H2O was added into 10 mL of distilled water using a magnetic stirrer, and then 125 mg (0.35 mmol) of EDDH was dissolved into the solution. Salem et al. studied the effects of pH on zeta potentials and reported that the higher the pH, the less the zeta potential of NPs. Since more negative NPs are more capable of penetrating cell membranes, pH was maintained at an alkaline level [14]. The zeta potential of b4 NPs was −6.35 mV. After being vigorously stirred for 10 min, the mixture was placed into a Teflon-lined autoclave vessel of 25 mL capacity, which was heated up to and maintained at 120°C for 30 h, and then was left to cool to room temperature. The dispersions were purified by centrifugation-redispersion cycles, and then the products were thoroughly washed with deionized water and absolute ethanol and were finally dried in a vacuum oven.

The antifungal activity of copper NPs was examined on the basis of in vitro inhibition of colony formation using Petri dish assay with minor modifications [17]. Phytopathogenic mycelial fungi including A. alternata, A. solani, Fusarium oxysporium, and a Penicillium sp. were cultivated on potato dextrose agar (PDA) and incubated at 28°C for 10 days. Conidia were collected from the cultures and homogenously suspended in sterile deionized water containing 0.1% Tween 80 and diluted to obtain the final concentration of 106 spores mL−1. About 900 μL of the conidial suspension was mixed with 100 μL of the obtained copper NPs, Bordeaux mixture, and deionized water to provide a final volume of 1 mL. About 10 μL samples of the mixtures were taken at 0, 1, 3, and 6 h after exposure time and then diluted 100 folds with sterile deionized water. About 50 μL aliquots of these diluted suspensions were spread on PDA plates, which were incubated at 28°C for 2 days, and then the numbers of survivals were counted as colony-forming unit per milliliter. All of the experiments were conducted in duplicates.

RESULTS AND DISCUSSION

In order to synthesize copper NPs, Cu(CH3CO2)2.H2O and EDDH were added to 10 mL deionized water. The pH of the solutions was set at 14 by using the sodium hydroxide (4 M) solution. To assess the effects of such factors as temperature, concentration, molar ratio, and reaction time on the morphology of the particles, several samples were prepared. By varying the EDDH concentration and making other changes in the mentioned parameters, we can control the particle size and shape. The process of sample preparation is summarized in Table 1. The XRD spectra of the particles showed that the product was metallic copper. Figure 1 shows the general XRD pattern of the produced copper particles was consistent with the standard
XRD patterns of copper (JCPDS 04-0836). All the diffraction peaks were related to the face-centered cubic phase of magnetite (fcc space group Fd 3m). In addition, the EDX analysis demonstrated that the copper NPs were pure, as shown in Fig. 2. On the basis of these results, it can be concluded that the pH value must be maintained at an alkaline level to produce metallic copper NPs. Also, the zeta-potential measurements showed that copper NPs have a negative surface charge (−6.35 mV) and can be used in biomedical applications.

The results suggest that the EDDH acts as both a reducing and a capping agent. In this synthesis process, the first Cu cations seem to coordinate with EDDH, and after the addition of alkali solution, the hydroxide groups seem to attack EDDH molecules and release the hydrazine molecules. The remaining chemical appears to be EDTA. Hydrazine seems to play a reducing role and EDTA as a capping agent in this reaction. The reaction is shown below (Scheme 1).

In an aqueous solution, EDTA forms an octahedral complex with Cu$^{2+}$ at pH = 7, which has a UV-Vis absorption spectrum of $\lambda_{\text{max}} = 740$ nm, as shown in Fig. 3. Similarly, Cu$^{2+}$ and EDDH can form an octahedral complex with the same absorption rate, and a complexation process similar to that of [Cu(EDTA)] can be expected. However, there are four nitrogen atoms in the EDDH molecule instead of the oxygen atoms in the EDTA molecule that participate in the complexation of Cu$^{2+}$. Maintaining the pH at 14 by adding the NaOH solution leads the $\lambda_{\text{max}}$ of the [Cu(EDDH)] complex solution to fall from 740 to 700 nm. This shift may have also occurred because of the EDDH amide arms hydrolyzed by OH$^-$ groups. The spectral shift may also show the sum of UV-Vis absorptions of all the molecules in the environment. By the half-time of the hydrothermal reaction, the UV-Vis spectra of the solution showed a new $\lambda_{\text{max}}$, which was consistent with those of the Cu$^{2+}$ + NH$_4^+$ + EDTA solution. Therefore, the proposed mechanism for the hydrothermal reaction and reducing Cu$^{2+}$ to CuO is the oxidation of hydrazine to NH$_4^+$ according to the known oxidation reaction, as shown below (Scheme 2).

Table 1 summarizes all the experiments and results. At the first stage, four samples are prepared at 120°C, and the products (Fig. 4, a$_1$) are spherical NPs ranging between 70 and 100 nm in size. In the next experiment, the reaction time is doubled, and the obtained products have branching and puffy structures ranging between 200 and 300 nm in size (Fig. 4, a$_3$). Increasing the time leads to the growth of spherical particles in different directions. (With time, spherical particles grow...
This growth increases the surface-to-volume ratio of the particles. By increasing the concentration of Cu$^{2+}$ in the a2 test, larger and okra-like particles have the opportunity for further growth, and the increase in EDDH concentrations leads to new formations around the growing particles in the reaction environment. Figure 4 (a2) shows the SEM images of these NPs. When the reaction time of test a2 increases from 30 to 60 h, the resulting particles collapse and the decomposed products are arranged in a spherical shape (Fig. 4, a4). The Van der Waals force determines the final arrangements of the particles. At the second stage, the reaction temperature is increased to 170°C, which leads to significant changes in the morphology of the particles. The conditions applied in the b1 test result in the formation of particles with regular geometric shapes (Fig. 5, b1). At low concentrations, a limited number of initial cores are formed. This increases the chance of a regular multidimensional growth. Increasing the reaction time to 60 h leads to the formation of filamentous particles (Fig. 5, b2). These particles start to grow on the surface of the coarser
particles when the initial concentration is reduced. Figure 5 (b3) shows that the products of the b3 test are composed of smaller particles. In fact, the much larger spherical particles are colonies of nanometer particles. The results of the test suggest that when the concentration of precursors is high, in the early stages of the reaction, the majority of initial copper cores are formed. Moreover, as a result of the high concentration of EDDH, spherical micelles are formed around the copper NPs. Hence, the new formation leads to the creation of a spherical template [19]. Further, the Van der Waals forces between the particles keep them together [20].

Out of the various copper NPs produced, optimal particles were examined for their antifungal properties (Fig. 5, b4). To examine the antifungal activity of copper NPs, prevention from germination and growth inhibition of a number of pathogenic fungi were assessed. The fungal conidia provide the latent forms of life and show more resistance to harsh conditions than those of vegetative forms of fungi (Fig. 6 and Fig. 7). The copper NPs react in the enzyme of the plant and inhibit the metabolism of the fungus. This effect has many sites of attack so the fungi rarely build a resistance to copper based fungicide. The antifungal activity of the copper NPs may have resulted from their interaction with protein molecules, which results in the inactivation of protein molecules, and also from their direct interaction with DNA molecules. This interaction may have caused a mutation in the DNA, and hence the cessation of its replication ability [14, 21, 22]. In addition, copper NPs can easily penetrate the cell wall (cell membrane), as these particles are considerably small in size. Particle accumulations in the cell membrane may lead to cell lysis [24, 25]. The antifungal activity of copper NPs may also have been caused by the disruption of transmembrane energy mechanism. Finally, the formation of insoluble compounds in the cell wall (cell membrane) may have disrupted the electron transport chain of the membrane.

![Figure 5.—SEM images of copper samples at 170°C.](image)

**Figure 5.—** SEM images of copper samples at 170°C.

![Figure 6.—Colony formation of the fungi affected by the CNP (●) and Bordeaux mixture (■). (a) A saprophytic strain of *Penicillium* sp., (b) *Fusarium oxysporum*, (c) *Alternaria alternata*, and (d) *Alternaria solani*.](image)
CONCLUSION

The synthesis and characterization of copper NPs in the presence of EDDH as a capping and reducing agent under hydrothermal condition were investigated and the effects of concentration ratios, reaction temperatures, and reaction time on NP formation were studied. The more uniform, smoother, more flexible, and more stable NPs were examined for their antifungal effects, and the results indicated that copper NPs have a greater antifungal activity than that of better-known antifungal agents, such as Bordeaux mixture.

ACKNOWLEDGMENT

The authors would like to gratefully acknowledge the support provided by the Research Institute of Petroleum Industry (RIPI) in producing synthesized EDDH. The authors would also like to express their gratitude to Dr. Ali Mahmoudi, Dr. Saeed Dehghanpour, and Dr. Khodadad Nazari for their practical assistance. Thanks are also due to our English editor Abolhassan Tajfar for his assistance with the manuscript.

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FIGURE 7.—Growth inhibition of the phytopathogenic fungi exposed to the CNP and Bordeaux mixture. (a) A saprophytic strain of Penicillium sp., (b) Fusarium oxysporum, (c) Alternaria alternata, (d) Alternaria solani. 0 (top) and 6 h (down) post-exposure. (Left) CNP. (Middle) Control. (Right) Bordeaux.
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