

Copper Nanoparticles Synthesized in Biopolymer Matrix and Their Application in Antibacterial Activity

Aminu Musa¹ , Ameen Hadi Mohammed *2⁰, Mansor Ahmad ³ , Monday Musah ⁴

¹Department of Pure and Industrial Chemistry, Faculty of Natural and Applied Sciences, Umaru Musa Yar'adua University, Katsina, Dutsin-ma Road, P.M.B 2218, Katsina 820001, Nigeria.

²Department of Chemistry, College of Science for Women, University of Baghdad, Baghdad, Iraq.

³Department of Chemistry, Faculty of Science, University Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia. ⁴Department of Chemistry, Faculty of Natural Sciences, Ibrahim Badamasi Babangida Univer–sity, Lapai, Nigeria.

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Abstract

Copper is a cheaper alternative to various noble metals with a range of potential applications in the field of nanoscience and nanotechnology. However, copper nanoparticles have major limitations, which include rapid oxidation on exposure to air. Therefore, alternative pathways have been developed to synthesize metal nanoparticles in the presence of polymers and surfactants as stabilizers, and to form coatings on the surface of nanoparticles. These surfactants and polymeric ligands are made from petrochemicals which are non- renewable. As fossil resources are limited, finding renewable and biodegradable alternative is promising. The study aimed at preparing, characterizing and evaluating the antibacterial properties of copper nanoparticles. Copper nanoparticles were prepared using gelatin biopolymer, CuSO4.5H2O ions and hydrazine as stabilizer, precursor salt and reducing agent respectively. However, vitamin C and NaOH solution were also employed as an antioxidant and pH adjuster. The synthesized nanoparticles were characterized using UV-visible spectroscopy (UV-vis), copper thermogravimetric analysis (TGA), zeta potential measurements powder, X-ray diffraction (XRD), field emission scanning electron microscope and transmission electron microscope (TEM). The UV-visible absorption spectrum confirms the formation of the CuNPs, which showed maximum absorbance at 583 nm. Results obtained from TEM indicated a decrease in size of particle from a low concentration to high concentration of the supporting materials. The optimum concentration of gelatin was found to be 0.75 wt%. The supporting materials used for this synthesis are biocompatible and the obtained products are stable in air. The synthesized CuNPs display promising antibacterial activities against B. subtilis (B29), S. aureus (S276), S. choleraesuis (ATCC 10708) and E. coli (E266) as gram positive and negative bacteria respectively.

Keywords: Antibacterial activity, Biopolymer, Copper Nanoparticles, Gelatin, Matrix.

Introduction

Recently, researchers have focused their attention on discovering potentials of using copper nanoparticles as eventual antimicrobial agent because of higher cost of precious metals like silver and gold ¹. Copper nanoparticles preparation is considerably more difficult as compared with precious metal counterparts, since copper nanoparticles are completely reactive in aqueous solution and air is considered stable under these conditions. Copper nanoparticles when exposed to atmospheric air, agglomeration appears instantly because of surface oxidation ². To overcome this issue, an inert atmosphere, such as nitrogen or argon is utilized. In some cases, copper nanoparticles have been synthesized using inorganic solvent and surfactant. Furthermore, different techniques have been employed for the preparation of CuNPs, which are classified as physical and chemical methods. Among the aforementioned techniques, the chemical reduction method is an easy and rapid technique to synthesize stable metal nanoparticles. The study was aimed at preparing, characterizing and evaluating the antibacterial properties of copper nanoparticles. Copper nanoparticles were prepared using gelatin biopolymer, CuSO₄.5H₂O ions and hydrazine as stabilizer, precursor salt and reducing agent respectively. However, vitamin C and NaOH solution were also employed as an antioxidant and pH adjuster.

The significant advantages of utilizing inorganic nanoparticles as related to antimicrobial agents of organic origin which are stable at increased temperature and pressure, their capability for resisting severe activities, robustness as well as long shelf life. Many researchers reported the biosynthesis of Se nanoparticles using Gram positive Bacillus mycoides and Gram negative Stenotrophomonas maltophilia and tested for its antimicrobial activity ³⁻⁶. The result revealed that the SeNPs remained active at minimum inhibitory concentration against P. aeruginosa but clinical isolates of yeast species, C. albicans and C. parapsilosis were not inhibited. In another study, silver nanoparticles that were supported on rice straws showed effective antibacterial activities against S. aureus and E. coli based on diffusion technique ⁷. Activities were detected when the concentrations of silver nanoparticles increased and particle size were decreased on the rice straw. Similarly, silver and gold nanoparticles were synthesized with the plant extract ⁸, Menthe piperita (Lamiaceae). The result showed that the prepared



nanoparticles are active against E.coli and Staphylococcus aureus. AgNPs were synthesized in inter-lamellar external and space the of montmorillonite by a method of chemical reduction using NaBH₄⁹. By utilizing Mueller Hilton agar and the disk diffusion technique, Gram positive and Gram-negative bacteria were examined for antibacterial activity with various sizes of AgNPs. Particles with the least size were found to experience considerably higher antibacterial performance.

Studies revealed that copper nanoparticles have antimicrobial action against a wide spectrum of bacteria, for example, Bacillus subtilis. Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella chloleraesuis, Candida albicans. *Escherichia coli*, and so forth ¹⁰⁻¹². The mechanism for the action of copper is not yet completely tacit. That is, copper nanoparticles could join to the bacterial cell layer, creating structural changes or utilitarian harms and hinder their development, up to the death of a cell ¹¹. A more vital instrument of activity been suggested for dissemination of the potential of cell membrane; because of cell filament development and reactive oxygen species could harm bacterial DNA and the cell membrane of bacteria, which could bring about protein oxidation and may result in death of bacterial cell ¹³. Larger surface-to-volume ratio copper nanoparticles offer more effective techniques for antibacterial action ¹⁴. Most of these investigations studied the efficiency of CuNPs in bacteria-killing through comparing the materials at diverse particle sizes or via comparing bacteria species. For various instance. Venkatakrishnan reported that the diameter of inhibition zones around the disk that contains CuNPs differs for various species of bacteria ¹⁵. The change in inhibition zone between different species of bacteria can be ascribed to dissimilar in the composition of cell wall. In another work, Tantubay reported the synthesis of spherical CuNPs stabilized by CMC and tested against a fungal (Candida tropicalis) and bacteria (Escherichia coli) through various methods and microscopic observation. The result showed very good activity for E. coli and C. tropicalis. In another study, CuNPs were synthesized with citron juice (citrus medica Linn) as reducing and stabilizing agent respectively ¹⁶.

Kirby-Bauer disk diffusion method was used to examine the antimicrobial activity of some bacteria species and plant pathogenic fungi which showed significant activity against *E.coli* followed by *S. typhi*, *P. aeruginosa*, *P. acnes*, and *K. pneumoniae*. Moreover, *F. culmorum* was the most sensitive of the examined fungi before *F. graminearum* and *F. oxysporum*, respectively. Furthermore, CuNPs stabilized by pectin biopolymer has been reported and used as disk diffusion approach, against Gram negative and Gram positive microorganisms ¹⁵. The antibacterial action displayed by CuNPs towards the

Materials and Methods

Materials

Chemical reagents utilized in the study were CuSO₄ .5H₂O (purity 99 %) purchased from Bendosen Laboratory Chemicals, ascorbic acid (purity 90 %) was purchased from Hamburg, hydrazine hydrate (35 % hydrazine) and NaOH (99 %) were procured from MERCK (Germany), while ethanol and Gelatin (type B) were bought from Sigma Aldrich (USA). Four species of bacteria were used in this study, including Gram-positive (*Bacillus subtilis* B29 and *Staphylococcus aureus* S276) and Gramnegative bacteria (*Salmonella choleraesuis* ATCC 10708 and *Escherichia coli* E266). Analytical grade reagent and deionized water were used for preparation of solutions.

Preparation of Copper Nanoparticles in Gelatin

Copper nanoparticles were prepared in gelatin, using the method previously reported ^{17,18}. 0.38 g of gelatin in volumetric flask containing 50.0 cm³ distilled water warmed to 40 °C in order to obtain 0.75 % (w/v) suspension. Then 15.0 cm^3 of CuSO₄.5H₂O (0.1 M) was added to 35 cm³ of the 0.75 % (w/v) gelatin suspension to obtain the 0.03 M final concentration. Thereafter, 2.5 cm³ of 0.02 M of ascorbic acid was added at 80 °C for 20 minutes with constant stirring. This was then followed by the addition of 5.0 cm³ solution of NaOH, with further mixing until a solution of light green colour was obtained. 2.5 cm³ of 35 wt % hydrazine was finally added to facilitate the reduction of copper ions. Within 30 min of the reaction, colour of the solution changed from dark to reddish brown at constant stirring. The CuNPs/gelatin was separated



tested Gram positive and Gram-negative bacteria was good and comparable to those of ofloxacin and kanamycin respectively. Antibacterial action of copper nanoparticles hydrosol has been investigated against *E.coli*, staphylococcus aureus. Also, the current study focuses on how effective it is against candida albicans ¹⁰. The measurements of microbial reduction were done as function of CuNPs after two hours of contact. Morphological and surface and alteration of strains which were exposed to CuNPs were studied at cellular level using atomic force microscope.

by centrifuging at 14,000 rpm for 10 minutes and kept at 60 °C in a vacuum overnight to dry.

Antibacterial Test

The test was carried out by placing the paper disc having a diameter of 6.0 mm, which contains 10 μ L of the sample suspensions, was placed on a nutrient broth agar plate inoculated with bacterial. Standard bactericidal agents for both positive and negative inhibitory controls were streptomycin 100 mg/L. The standard for bacterial inoculum was 0.5 MF units, or 108 colony-forming units of each bacterium added on a plate. After being inoculated, the plates were then incubated and inverted at a temperature of 37 °C for 24 hours under UV light, and then the zone of inhibition was measured using millimeter units to the nearest whole number. The entire tests are repeated in triplicate.

Characterization

Different characterization techniques were employed to characterize CuNPs stabilize by gelatin.

Ultraviolet-Visible Absorption Spectroscopy

UV-vis absorption spectroscopy is an important spectroscopic method used for the characterization of metal nanoparticles. Given the unique surface plasmon resonance exhibited by specific metals and their corresponding oxides (Cu, Ag, Au, and Pt), the technique becomes important for early detection of the presence of nanoparticles. In this investigation, the SPR bands of the produced nanoparticles were detected using a UV-Visible spectrophotometer, model UV 1650 PC-Shimazu B. The prepared

sample was poured into a quartz cuvette at a volume of around 70.0 L. Spectra were run within the range of 300 and 1000 nm.

Thermal Gravimetric Analysis

Thermal behavior of the prepared samples was investigated by using thermal gravimetric analysis (TGA) and recorded with a thermogravimetric analyzer TGA 7 (Perkin Elmer) in the temperature range of 35 to 600 °C at constant heating rate of 10 °C/min and continuous nitrogen flow of 20 mL/min. 15 mg of the sample weight was used. As a function of temperature, sample weight loss was measured.

Zeta Potential Analysis

In the Malvern Zeta Instrument 3000 (UK model of the Malvern instrument), zeta size measurements and zeta potential were performed on the prepared samples 19,20 . In this procedure, the cuvette was filled with 100 samples that had been re-suspended in 900 mL milli Q water. The measurement was carried out at 25 °C and a scattering angle of 90°. Dielectric constant was 78.5 while refractive indexes and material dispersion were set at 1.330 and 1.365 (viscosity (CP) 0.8872), respectively.

X-ray Diffraction

To determine material's crystal structure and crystallinity, an X-ray diffraction analysis is

Results and Discussion

Characterization

UV-Visible Spectroscopy

The UV-visible absorption spectrum of samples is shown in Fig 1 A. The generated UV-visible spectra for gelatin matrix supported CuNPs show the development of CuNPs with the highest wavelength at around 583 nm. Fig 1 A (a-e) indicates that there was a gradual increase in the intensity of the SPR peak position as the concentration of gelatin was increased from 0.1 to 1.0 wt% (a-e). Increasing the absorbance indicated an increase in the concentration of CuNPs. Also, Fig 1(a-b) shows the blue shift in SPR position from 600 nm to 592 nm, which is an indication of particle size decrease, as a result of an increase in absorbance caused by an increase in gelatin concentration. A gradual increase in the size of CuNPs and strong inter-micelle interaction were caused by a red shift in SPR from 583 nm to 590 nm.

Baghdad Science Journal employed. The structure of the produced CuNPs/gelatin was examined using Philip X'pert PRO diffractormeter (PANanalytical, Almedo, Netherlands). The instrument was run at 30 mA and 40 Kv, and the X-ray beam was nickel-filtered Cu (= 1.542°A). The temperature range of the scanning scope of 2θ was 5 to 80°, and the scan speed was

Transmission Electron Microscopy

 $2^{\circ}/\text{min}$.

Transmission Electron Microscopy (TEM) was used in investigating the shape and the size of the produced materials. At room temperature, the 120 Kv H-7100 transmission electron microscope (TEM Hitachi, Japan) was employed. Particle size distribution was determined with aid of UTHSCSA Image Tool, version 3.0. By dropping a portion of the solution onto a copper grid that had been covered with carbon, the sample was prepared.

Field Emission Scanning Electron Microscopy

Field Emission Scanning Electron Microscopy was employed for observation of the morphology of the samples. It was carried out using a Jeol JSM-7600F field emission scanning microscope from Echingen, Germany. With the help of the Baltec SCD005 Sputter-coater (Bal-tec. Canonsburg, USA), carbon tape was used to mount the samples to the aluminum stubs before the samples were sputtered with gold for 30 minutes at 20 mA.



Figure 1. UV-visible absorption spectra of CuNPs/Gelatin with different concentrations of gelatin: (a) 0.10, (b) 0.25, (c) 0.50, (d) 0.75 and (e) 1.00 wt%

Thermal Gravimetric Analysis

Thermal property is an important characteristic of materials and polymers ²¹⁻²³. The thermal properties





of the gelatin with those of CuNPs/Gelatin were investigated using TGA. TGA and DTG thermograms, given in Fig. 2 (A-B) display the degradation behaviors of gelatin and CuNPs at different concentrations 0.1, 0.25, 0.5, 0.75 and 1 wt % of gelatin. Results obtained showed that, for all samples, there was initial loss of weight initial weight loss at temperatures below 100 °C. This could be due to loss of moisture from the surface. The degradation of gelatin at the initial stage occurred around 329 °C, and the thermal decomposition residue of gelatin at 538 °C was 24

wt% as shown in Fig 2 [A-B (a)]. The present results are in agreement with Makabenta, who observed similar behavior for gelatin. Similarly, the result indicates that the increased in gelatin quantity lead to increase in the thermal stability of the gelatin, which is majorly due to higher heat stability of metallic copper. In the final thermal destruction, residual weight percentages for CuNPs/Gelatin were 90, 91, 93 and 95 wt%, for 0.1, 0.25, 0.5, 0.75 and 1 wt % respectively. The high residual content CuNPs/Gelatin at 1 wt. % is certainly attributed to the high formation of CuNPs in the gelatin.



Figure 2. (A) TGA thermograms and (B) DTG thermograms of Gelatin (a), and CuNPs/Gelatin at different concentrations of Gelatin (b) 0.10, (c) 0.25, (d) 0.50, (e) 0.75 and (f) 1.00 wt%.

Zeta Potential Analysis

Zeta potential measurement was performed to determine charges and nanoparticle stability. Figure 3 depicts the zeta potential of CuNPs/gelatin in water that was neutral. Samples exhibited negative zeta potential value of -37.9 ± 0.6 mV in aqueous solution. The negative potential value was due to the presence of amino and carboxylic groups on the CuNPs surface. Zeta potential is an important parameter that affects the stability of colloidal dispersion. Particles with high negative and or positive value than ± 30 mV for zeta potential are usually considered to give rise to stable dispersions²⁴. High concentration of gelatin stabilized CuNPs shows greater stability in aqueous dispersion.



Figure 3. Zeta potential of Gelatin and CuNPs/Gelatin

X-ray Diffraction

XRD analysis was employed to investigate the crystallographic nature of copper nanoparticle and gelatin prepared. As shown in Fig 4(a-b), the peaks

located at 43.31° , 50.54° and 74.15° can be obviously observed. These peaks can be indexed on the basis of face-centered cubic (fcc) phase of copper to (111), (200) and (220), crystallographic plane ¹²⁻²⁵. As depicted in Fig. 4(a), gelatin is responsible for the broad diffraction peak at 22.35°. The crystalline size of the prepared copper nanoparticle was found to be 16 nm. The average nanoparticles sizes obtained from XRD are nearly consistent with the result of TEM measurements. This result indicates there is no peak related to copper oxide nanoparticle.



Figure 4. XRD diffractogram for Gelatin (a) and CuNPs/Gelatin



Morphology

The TEM micrograph provides useful information on shape and size of nanoparticles. The TEM image of copper nanoparticle and histogram showing particles size distribution is shown in Fig 5. The image indicates that the shape of nanoparticles studied is spherical. The histogram in Fig 5 represents the size distribution of the particle; the average particle size was 2.17 ± 1.12 nm. This highlights the importance of gelatin in controlling the nanoparticles size as reported by Mahmoudi ²⁶. These results coincide with the UV-vis absorption analysis reported earlier. The FESEM micrograph of gelatin and CuNPs in gelatin concentration of 0.75 wt %, are exhibited in Fig 6 (a-b). The obtained micrograph for the CuNPs/gelatin, specify that the CuNPs are surrounded within the matrix of the gelatin. The particles have the different size and patterns of distribution that are based upon the gelatin concentration.



Figure 5. TEM Image and histogram presenting particle size distribution of CuNPs/Gelatin





Figure 6. FESEM micrographs of Gelatin (a) and CuNPs/Gelatin

Antibacterial Activity

antibacterial activity of prepared The the CuNPs/Gelatin was studied. Susceptibility was determined through measurement of zone of inhibition. The DIZ is a measurement that reveals the extent of susceptibility of the testing bacteria. Inhibition zone values were obtained for all the aqueous solutions of CuNPs/Gelatin against Grampositive; S. aureus (S276), B. subtilis (B29) and Gram-negative; S. choleraesuis (10708) and E.coli (E266). The images and results of the inhibition zones are presented in Fig 7 and Table 2. The zones of inhibition were clearly seen in all the samples,

which signify activity of the prepared CuNPs in all the bacteria. Furthermore, the negative control which is the gelatin solution did not display any zone of inhibition this may suggest the lack of antibacterial activity of gelatin in this test. It is apparent from Table 1, that the diameter of inhibition zone is highest at the sample G4, which have 0.75 wt% concentration of gelatin as indicated in the Fig 8. This may suggest the amount and size of copper can control the antibacterial activity of the prepared sample. Fig 9 (a-b) indicates the correlation between zone of inhibition and the concentration of gelatin as the size controller of CuNPs.



Figure 7. Inhibition zones, images of CuNPs/Gelatin at different Gelatin concentrations (G1) 0.10, (G2) 0.25, (G3) 0.50, (G4) 0.75 and (G5) 1.00 wt% against bacteria (A1-A2) S276, (B1-B2) B29, (C1-C2) 10708 and (D1-D2) E266



Table 1. Inhibition zone data of CuNPs/Gelatin against different bacteria							
Bacteria	Diameter of inhibition zone (mm)						
	С	G0	G1	G2	G3	G4	G5
Staphylococcus aureus (S276)	30.0	10.0	9.5	11.5	11.0	16.0	7.0
Bacillus Subtilis (B29)	32.5	15.0	15.0	16.0	17.5	18.5	19.5
Salmonella choleraesuis (10708)	33.5	13.0	12.5	11.5	13.5	18.0	10.0
Escherichia coli (E266)	34.0	12.0	14.5	15.0	18.0	22.0	10.5



Figure 8. Diameter of inhibitory zones for CuNPs (G0) and CuNPs/ Gelatin using various gelatin concentrations (G1) 0.10, (G2) 0.25, (G3) 0.50, (G4) 0.75 and (G5) 1.00 wt% with control (C) antimicrobial agent (streptomycin)

Conclusion

In this study, copper nanoparticles stabilized by gelatin in aqueous solution under atmospheric air have been prepared successfully using the method, chemical reduction with gelatin concentration of 0.75 wt%. The prepared CuNPs/Gelatin was characterized using UV-vis, XRD, TEM, Zeta potential and TGA. Results from TEM and XRD showed that the mean diameters of CuNPs gradually decreased as the concentration of the gelatin increased. The CuNPs/Gelatin showed an

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Figure 9. Antibacterial activities of CuNPs/Gelatin for different (a) Particles sizes and (b) concentrations of gelatin

insignificant increase in thermal stability with the increasing concentration of gelatin. The results showed good antibacterial activity against tested bacteria. The best improved property of CuNPs/Gelatin was found at the 0.75 wt % gelatin concentration. Finally, the result showed a good correlation between the zone of inhibition and the concentration of gelatin as the size controller of CuNPs.

Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for

Authors' Contribution Statement

A.M. and A.H. carried out the design, acquisition of data, analysis, interpretation, and participated in the the manuscript. M.A. helped drafting in interpretation and analysis. M.M. contributed to **References**

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Baghdad Science Journal

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جسيمات النحاس النانوية المركبة في مصفوفة البوليمر الحيوي وتطبيقاتها في النشاط المضاد للبكتيريا

امينو موسى 1، امين هادي محمد²، منصور بن احمد³، ماندي موساح⁴

اقسم الكيمياء الصناعية، كلية العلوم الطبيعية والتطبيقية، جامعة عمر موسى، كاتسينا، نايجيريا

² قسم الكيمياء، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق 3 قسم الكيمياء، كلية العلوم، جامعة بوترا الماليزية، سيلانكور، ماليزيا

مسم الميميون مين المعوم، جمعت بوتر، المعايرية ميوريون معيري 4 قسم الكيمياء، كلية العلوم الطبيعية، جامعة ابر اهيم بادماسي، لاباي نايجيريا

الخلاصة

النحاس هو بديل أرخص من العديد من المعادن النبيلة مع مجموعة من التطبيقات المحتملة في مجال علم النانو وتكنولوجيا النانو. ومع ذلك ، فإن جسيمات النحاس النانوية لها قيود كبيرة ، والتي تشمل الأكسدة السريعة عند التعرض للهواء. لذلك ، تم تطوير مسارات بديلة لتجميع الجسيمات النانوية المعدنية في وجود البوليمرات والمواد الخافضة للتوتر السطحي كمثبتات ، ولتشكيل الطلاءات على سطح الجسيمات النانوية. هذه المواد الخافضة للتوتر السطحي والروابط البوليمرية مصنوعة من بتروكيماويات غير متجددة. نظرًا لمحدودية الموارد الأحفورية ، فإن إيجاد بديل متجدد وقابل للتحلل البيولوجي يعد أمرًا واعدًا ، حيث هدفت الدراسة إلى إعداد وتوصيف وتقييم الخصائص المضادة للبكتيريا لجسيمات النحاس النانوية. تم تحضير جسيمات النحاس النانوية باستخدام البوليمر الحيوي الجيلاتيني ، أيونات CuSO4.5H20 والهيدر ازين كمثبت ، ملح سلائف و عامل اختزال على التوالي. ومع ذلك ، تم استخدام محلول فيتامين C و أيونات CuSO4.5H20 والهيدر ازين كمثبت ، ملح سلائف و عامل اختزال على التوالي. ومع ذلك ، تم استخدام محلول فيتامين C بي أيونات CuSO4.5H20 والهيدر ازين كمثبت ، ملح سلائف و عامل اختزال على التوالي. ومع ذلك ، تم استخدام محلول فيتامين C و أيونات CuSO4.5H20 والهيدر ازين كمثبت ، ملح سلائف و عامل اختزال على التوالي. ومع ذلك ، تم استخدام التحليل الطيفي المرئي بي المقعة فوق البنفسجية (UV-vis) والهيدر ازين كمثبت ، ملح والوزني (TEM) ، مسحوق قياسات زيتا المحتملة ، حيود الأشعة السينية عدوينا CuNPs أيضًا كمضاد للأكسدة وضبط درجة الحموضة. تم تمييز جسيمات النحاس النانوية المرئي للأشعة فوق البنفسجية عائر وني لمسح الانبعاث الميداني والمجر الإلكتروني النافذ (TEM) ، يؤكد طيف الامتصاص المرئي للأشعة فوق البنفسجية ما ما المجر الإلكتروني لمسح الانبعاث الميداني والمجهر الإلكتروني النافة (TEM) . يؤكد طيف المرئي الحصول عليها من التولي للائمة فوق البنفسجية وعرين درالامة للبركيز المنخفض إلى التركيز العالي للمواد الداعمة. وجد أن التركيز الأمثل للجيلاتين هو 7.0. حم الجسيمات من التركيز المنخفض إلى التركيز العالي للمواد الداعمة. وجد أن التركيز الأمثل للجيلاتي الوزن. المواد وحم الجسيمات من التركيز المنخفض إلى الموادة الحاصة وحد أن التركيز الأمثل للجيلاتي مو 7.0. والداعمة المستخدمة في هذا التركيب متوافقة حيوياً والمنت

الكلمات المفتاحية: النشاط المضاد للبكتيريا ، البوليمر الحيوي ، جزيئات النحاس النانوية ، الجيلاتين ، المصفوفة.