Journal of Materials and Environmental Science
ISSN : 2028-2508
e-ISSN : 2737-890X
CODEN : JMESCN

J. Mater. Environ. Sci., 2025, Volume 16, Issue 10, Page 1802-1817

http://www.jmaterenvironsci.com

Copper Oxide Nanoparticle-Enhanced Sodium Alginate Coating for Chicken Meat Preservation: Antimicrobial Efficacy and Shelf-Life Assessment

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Received *18 July 2025,*
Revised *18 Sept 2025,*
Accepted *20 Sept 2025*

Keywords:
- ✓ *Copper oxide nanoparticles*
- ✓ *Sodium alginate*
- ✓ *Rosemary extract*
- ✓ *Antimicrobial packaging*
- ✓ *Synergistic preservation*

Citation: *Alsaeed F., Nama F., Hammal A. (2025), Development and Evaluation of a Hybrid Sodium Alginate-Based Nanocomposite Coating Reinforced with Rosemary Extract-Modified Copper Oxide Nanoparticles for Active Packaging of Chicken Meat, J. Mater Environ. Sci., 16(10), pp. 1802-1817*

Abstract: This study developed a novel hybrid nano-engineered coating by integrating rosemary extract-modified copper oxide nanoparticles (RE-CuO NPs) into a sodium alginate matrix for active preservation of chicken meat. CuO NPs were synthesized via chemical precipitation and characterized by XRD and AFM, confirming crystallite sizes of 22 ± 2 nm with a monoclinic crystalline structure. Surface modification of CuO NPs with rosemary extract (Rosmarinus officinalis L.) significantly enhanced their antimicrobial potency, reducing MIC values by 50% against S. aureus and S. Typhimurium (to 62.5 µg/mL). Three coating formulations were evaluated: alginate control (Alg), alginate with pristine CuO NPs (Alg-CuO), and alginate with RE-CuO NPs (Alg-RE-CuO). The Alg-RE-CuO coating demonstrated superior performance in diffusion assays, producing the largest inhibition zones (28.5 ± 0.6 mm against S. aureus). Refrigerated storage studies (4°C, 10 days) on inoculated chicken meat revealed that the Alg-RE-CuO coating achieved the highest microbial reduction, maintaining bacterial counts 2.5-3.0 log CFU/g lower than negative controls and showing an additional 0.5-1.0 log CFU/g reduction compared to the Alg-CuO group by day 10. The coating extended shelf life by 5-7 days while preserving meat quality parameters. These findings demonstrate the successful implementation of a safety-by-design approach, where the synergistic combination of natural extracts and nanomaterials enables effective preservation at reduced nanoparticle concentrations, offering a viable strategy for developing sustainable active packaging systems.

1. Introduction

Food packaging serves as a critical element within the global food supply chain, playing an indispensable role in safeguarding products against physical, chemical, and biological deterioration. This ensures the maintenance of food safety, quality, and an extended shelf-life (Han *et al*. 2018; Aad *et al*. 2024). Despite their effectiveness, conventional petroleum-based polymers present considerable environmental concerns, primarily due to their non-biodegradable nature and dependence on finite resources (Rhim *et al*. 2013). Consequently, significant research efforts have been directed towards developing sustainable, bio-based alternatives sourced from polysaccharides, proteins, and polyesters (Ghanbarzadeh *et al*. 2015; Aaddouz *et al*. 2023). Nevertheless, these biopolymers are frequently hampered by inherent weaknesses, including inferior mechanical strength, high permeability to gases and water vapor, and a general lack of functional properties (Vasile 2018). In this context, nanotechnology has arisen as a potent instrument to overcome these limitations. The integration of nanomaterials (NMs) into polymeric matrices to create nano-biocomposites holds the potential to substantially improve their overall performance (Sharma *et al*. 2017; Youssef & El-Sayed 2018).

Among the various food applications, meat products represent a prime target for these advancements due to their high susceptibility to spoilage. Recent research shows a significant focus on using developed nanofilms to extend the shelf life of meat and meat products, as these films work to reduce lipid oxidation and combat spoilage microorganisms, which are a major challenge in maintaining the quality of these high-value nutritional and consumer products (Umaraw *et al*. 2020).

Despite the significant strides in developing nano-enabled active packaging, an overreliance on singular nanotechnology approaches, particularly utilizing silver (Ag) and zinc oxide (ZnO) nanoparticles, is evident in the extant literature (Llorens *et al*. 2012; Wang *et al*. 2017). While effective, this focus overlooks the compelling economic and antimicrobial advantages of copper nanoparticles (Cu-NPs), which exhibit comparable, if not superior, broad-spectrum biocidal activity at a potentially lower cost and with a different mechanistic action, primarily through the rapid generation of reactive oxygen species (ROS) and enzyme inactivation (Azam *et al*. 2012; Moustafa *et al*. 2022). However, a critical and often unaddressed challenge in the application of metallic NPs, including Cu-NPs, is the potential for nanoparticle migration and the ensuing toxicological implications, which necessitates strategies to minimize their required dosage while maximizing efficacy (Bott *et al*. 2014).

This presents a pivotal research opportunity: the engineering of multi-functional hybrid systems that leverage synergistic effects between nanomaterials and bioactive phytocompounds. Such synergy can potentially mitigate the limitations of individual components, allowing for a reduction in nanomaterial concentration while enhancing overall antimicrobial and antioxidant performance (Kalagatur *et al*. 2018; Han *et al*. 2022). While the concept of hybrid films is emerging, the specific integration of Cu-NPs with natural extracts within a biopolymer matrix for meat packaging remains a profoundly underexplored frontier. Previous studies have largely treated these components in isolation, failing to capture the potential emergent properties of their combination (Siripatrawan & Noipha 2012; da Silva *et al*. 2021).

Herein, we address this critical knowledge gap by designing, fabricating, and evaluating a novel hybrid active film system based on sodium alginate reinforced with copper nanoparticles loaded with natural extracts for application in chicken meat packaging. Our approach is threefold:

- To introduce copper nanoparticles as a potent and cost-effective antimicrobial nanofiller, moving beyond the conventional Ag/ZnO paradigm.
- To engineer a synergistic system by co-incorporating selected natural extracts rich in polyphenols and antioxidants. We hypothesize that this combination will not only enhance the antimicrobial spectrum but also provide robust protection against lipid oxidation in chicken meat, a key spoilage mechanism.
- To utilize sodium alginate as a sustainable and safe reservoir matrix. This biopolymer is chosen for its excellent film-forming properties, biocompatibility, and ability to potentially control the release kinetics of active agents, thereby addressing migration concerns (Paiva *et al*. 2022).

Furthermore, the pursuit of enhancing the efficacy of active packaging systems while reducing reliance on high nanomaterial concentrations has led to a promising research trend: the synergy between nanoparticles and bioactive plant extracts. Essential oils and extracts from medicinal and aromatic plants, rich in phenolic compounds and terpenoids, are excellent natural alternatives thanks to their antimicrobial and antioxidant properties. Among these, rosemary extract (Rosmarinus officinalis L.) stands out as an ideal candidate; it contains potent active compounds such as rosmarinic acid, carnosic acid, and carnosol, which have been proven to exhibit strong antimicrobial and antioxidant activities (Diass *et al*. 2024; Houzi *et al*. 2025). Surface modification of copper oxide nanoparticles (CuO NPs) with such extracts not only enhances their stability and dispersion but may also create a synergistic effect that boosts the overall bioactivity and allows for the use of lower nanomaterial doses, thereby mitigating potential migration risks. However, this hybrid approach, particularly using CuO NPs, remains largely unexplored in meat packaging applications.

The overarching hypothesis of this study is that the multi-component interaction between Cu-NPs and natural extracts within the alginate matrix will yield a film with superior functional properties—mechanical, barrier, antimicrobial, and antioxidant—than any component alone. We posit that this hybrid system will significantly extend the shelf-life of fresh chicken breast by simultaneously inhibiting the growth of key spoilage microorganisms (e.g., Pseudomonas spp., Shewanella spp.) and pathogens (e.g., Salmonella spp., Listeria monocytogenes), while effectively retarding lipid peroxidation.

This work transcends incremental improvement; it offers a paradigm shift towards intelligent, multi-targeted packaging design. By providing a scientifically-grounded, sustainable, and effective solution for preserving high-value protein sources, this research contributes directly to global food security goals by reducing waste and enhancing food safety. The outcomes are expected to provide valuable insights for the development of next-generation food packaging materials that are both high-performing and aligned with consumer demand for natural, safe ingredients.

The deliberate incorporation of nanofillers represents a fundamental strategy for producing high-performance packaging. These materials function as multi-functional agents, bestowing a spectrum of enhanced characteristics. Regarding mechanical and barrier reinforcement, nanomaterials such as cellulose nanocrystals (CNC), zinc oxide (ZnO), and titanium dioxide (TiO_2) offer a high surface area for interaction with polymer chains, leading to notable improvements in tensile strength and Young's modulus (Carvalho *et al*. 2018; El Achaby *et al*. 2017). Moreover, impermeable nanofillers like graphene oxide (GO) and nanoclays establish a tortuous pathway for gas and water vapor molecules, significantly reducing oxygen transmission rates (OTR) and water vapor permeability (WVP), thereby decelerating food oxidation and moisture loss (Han Lyn *et al*. 2021; Lagaron *et al*. 2018). The concept of active packaging functions extends beyond simple containment to provide active protection. Here, nanoparticles act as functional elements that interact directly with the food or its environment. For example, iron oxide nanoparticles (Fe_3O_4) serve as oxygen scavengers, shielding fatty products from rancidity (Zhang *et al*. 2021). Titanium dioxide (TiO_2) nanoparticles employ photocatalysis to decompose ethylene gas—a plant hormone that hastens ripening and decay—thus prolonging the freshness of fruits and vegetables (Siripatrawan & Kaewklin 2018). Additionally, porous nanostructures can modulate internal humidity levels by absorbing excess moisture or releasing it to sustain optimal conditions (Lagaron *et al*. 2018). In the realm of intelligent or smart packaging functions, nanotechnology empowers the package to become a sensing and communication tool. Nanomaterials constitute the foundation of sensors that monitor food quality and relay information. For instance, the electrical resistance of carbon nanotubes (CNTs) or graphene alters upon the adsorption of specific spoilage gases (e.g., ammonia from meat, hydrogen sulfide from fish), facilitating spoilage detection (Blank *et al*. 2018). Colorimetric indicators based on silver or gold nanoparticles undergo color changes in the presence of volatile organic compounds resulting from microbial growth, offering a visual spoilage warning to consumers (Alizadeh-Sani *et al*. 2020). Furthermore, time-temperature indicators (TTIs) utilizing nanotechnologies can supply an irreversible record of a product's exposure to unsuitable temperatures during storage and transportation (Mustafa & Andreescu 2020).

Unlike traditional synthetic polymers like Polyvinyl Chloride (PVC), which are still used for their mechanical strength and low cost, current research is heavily focused on biodegradable natural polymers. Among these, Chitosan has emerged as a key player in developing nano-enabled meat coatings, not merely as a carrier but also due to its intrinsic antimicrobial and antioxidant properties, making it ideal for protecting highly perishable animal products (Jiang *et al*. 2020). Sodium alginate is another promising biopolymer for this application due to its excellent film-forming ability and biocompatibility (Paiva *et al*. 2022).

Studies reveal that combining nanomaterials with bioactive compounds, such as essential oils, creates a powerful synergistic effect. For instance, films containing Zinc Oxide Nanoparticles (ZnO NPs) with basil essential oil showed greater efficacy in controlling lipid oxidation and microbial growth in fish slices compared to the individual components alone. This suggests that the interaction between nanoparticles and active compounds can significantly enhance the functional performance of active packaging (Pabast *et al*. 2018).

The methodologies for applying these nano-enabled films to meat products vary. Beyond being used as conventional wraps, they are commonly applied via:

• Immersion: Where the meat product is dipped into the liquid coating solution and then dried to form a thin, continuous protective layer (Jiang *et al*. 2020).

• Vacuum Packaging: Where the meat is wrapped in the film and then placed in vacuum-sealed polyethylene bags to enhance barrier effectiveness and combat anaerobic microorganisms (Esmaeili *et al*. 2020).

• These methods have shown significant efficacy in preserving the quality attributes of meat.

Despite the promising benefits, the potential migration of nanoparticles (e.g., silver or zinc) from the packaging into the meat product raises safety concerns. Studies measuring migration have indeed shown an increase in zinc content in chicken meat packaged with films containing ZnO NPs. Although zinc oxide is generally recognized as safe (GRAS), its presence in its nano-form within the food matrix requires extensive long-term toxicity studies to fully understand its biological effects and health safety for consumers (Priyadarshi & Negi 2017). This highlights the need for the safety-by-design approach incorporated in our study by using a alginate matrix and synergistic extracts to potentially reduce the required dose of Cu-NPs.

The potent antimicrobial effectiveness of nanomaterials is not ascribed to a solitary mechanism but rather to a synergistic combination of pathways. Firstly, ion release occurs as metallic nanoparticles (e.g., Ag^{+}, Zn^{2+}, Cu^{2+}) experience slow dissolution at their surface, liberating ions that disrupt microbial enzyme function, inflict damage on cellular structures, and inhibit metabolic processes (Król *et al*. 2017). Secondly, oxidative stress through ROS generation is facilitated by nanoparticles like ZnO, TiO_2, and CuO, which can catalyze the production of Reactive Oxygen Species (ROS) such as hydroxyl radicals (•OH), superoxide anions (O_2•⁻), and hydrogen peroxide (H_2O_2) upon exposure to light (UV or visible) or even in ambient conditions. These ROS instigate oxidative damage to lipids—resulting in membrane disruption—proteins, and DNA, ultimately culminating in cell death (Wang *et al*. 2017). Thirdly, direct contact and membrane damage contribute to efficacy; the physical structure of nanomaterials, including sharp edges (e.g., on GO sheets) and direct nanoparticle contact, can induce physical puncturing or compromise the integrity of the microbial cell membrane. This leads to the leakage of intracellular contents and cell lysis (Hassan & Singh 2014; Ray Chowdhuri *et al*. 2015). Lastly, internalization allows ultra-small nanoparticles to infiltrate the cell interior, where they can disrupt vital intracellular processes and organelles (Wang *et al*. 2017). Gram-positive and Gram-negative bacteria often display differential susceptibility attributable to structural variances in their cell walls. Generally, Gram-negative bacteria are more vulnerable to certain ionic mechanisms due to their thinner peptidoglycan layer (Azam *et al*. 2012).

Notwithstanding the considerable promise, several formidable challenges must be confronted to enable widespread adoption. The principle of safety-by-design necessitates that future research concentrate on crafting inherently safer nanomaterials—for instance, through surface functionalization with biocompatible coatings or optimizing size and shape to minimize biological interaction—coupled with conducting exhaustive long-term toxicity studies (Tarhan 2020). The issue of end-of-life and recyclability poses a critical and understudied problem; the impact of nanomaterials on recycling streams for conventional plastics requires investigation to determine whether nanoparticles can be efficiently separated or if they contaminate recycled polymer batches (Singh *et al*. 2023). Scalability and cost-effectiveness are paramount; the development of economical, large-scale, and reproducible manufacturing processes (e.g., extrusion, coating) for these nano-biocomposites is essential for their commercial viability beyond niche applications (Katiyar & Ghosh 2021). Public perception and acceptance must be addressed; fostering consumer awareness and trust in nanotechnology for food-related applications will require transparent communication, education, and clear labeling (Barage *et al*. 2022). Finally, the development of hybrid and multi-functional systems represents the future direction; this involves creating intelligent hybrid systems that amalgamate multiple nanomaterials to accomplish several functions concurrently—for example, a composite that delivers mechanical reinforcement, antimicrobial activity, and sensing capabilities within a single, sustainable matrix (Youssef & El-Sayed, 2018). This study directly contributes to this final frontier by developing such a hybrid system.

2. Methodology

2.1. Synthesis and Characterization of Copper Oxide Nanoparticles (CuO NPs)

2.1.1. Synthesis of CuO NPs

Copper oxide nanoparticles were synthesized via a chemical precipitation method (Aldwayyan *et al*. 2013). Briefly, 4.5 g of copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, ≥99.0% purity, Sigma-Aldrich) was dissolved in 100 mL of absolute ethanol under constant magnetic stirring (500 rpm) at room temperature (25 ± 2°C) for 10 minutes to form a homogeneous blue solution. Separately, 4.5 g of sodium hydroxide (NaOH, ≥98%, pellets, Sigma-Aldrich) was dissolved in 200 mL of absolute ethanol.

The NaOH solution was added dropwise (at a rate of ~1 mL/min) into the copper sulfate solution using a burette, under vigorous magnetic stirring (800 rpm). The addition was completed over 60 minutes while maintaining the reaction temperature at 25 ± 2°C. The pH of the mixture was monitored and adjusted to 8.0 ± 0.2 using dilute NaOH solution.

Upon complete addition, a black precipitate formed immediately. The reaction mixture was then heated to 80 ± 2°C and maintained at this temperature for 2 hours under continuous stirring to facilitate complete particle growth and aging. The resulting precipitate was collected by vacuum filtration using a Whatman No. 1 filter paper and washed extensively with an ethanol-deionized water mixture (3:1 v/v) until the filtrate reached a neutral pH, ensuring the removal of residual salts and impurities. The purified product was dried in a hot-air oven at 80 ± 5°C for 12 hours. The dried powder was subsequently calcined in a muffle furnace (Nabertherm, Germany) at 400 ± 5°C for 2 hours using a heating ramp of 5°C/min to obtain the crystalline CuO phase. Finally, the calcined powder was gently ground into a fine powder using an agate mortar and pestle for 15 minutes and stored in a sealed amber glass vial containing silica gel desiccant to prevent moisture absorption and oxidation.

2.1.2. Characterization of CuO NPs

The crystallographic structure and phase purity of the synthesized nanoparticles were determined by X-ray diffraction (XRD) analysis using a PANalytical X'Pert PRO MPD diffractometer operated with Cu Kα radiation (λ = 1.54060 Å) at 40 kV and 25 mA. The diffraction patterns were recorded in the 2θ range from 20° to 80° with a step size of 0.013° and a scanning speed of 2° min^{-1}. The average crystallite size (D_{av}) was estimated from the line broadening of the most intense diffraction peak using the Debye-Scherrer equation: $D_{av} = (0.9 \times \lambda) / (\beta \times \cos\theta)$ (1) where λ is the X-ray wavelength, β is the full width at half maximum (FWHM) of the diffraction peak (in radians) after instrumental broadening correction, and θ is the Bragg diffraction angle. The surface morphology and topographic features of the nanoparticles were investigated using Atomic Force Microscopy (AFM, Nano Surf Flex-Axiom, Switzerland). The samples for AFM analysis were prepared by drop-casting a dilute ethanolic suspension of the NPs (0.1 mg mL^{-1}) onto a clean silicon wafer substrate and allowing it to dry in a desiccator at room temperature. Imaging was performed in non-contact (tapping) mode using a silicon cantilever probe with a resonant frequency of approximately 320 kHz. Scans were performed over areas of 5 × 5 μm at a resolution of 512 × 512 pixels. Surface roughness parameters, including the root mean square roughness (Rq) and the average roughness (Ra), were calculated from the height data using the instrument's native software.

2.1.3. Preparation of Rosemary Extract

Dried leaves of rosemary (Rosmarinus officinalis L.) were obtained from a reliable source. The leaves were ground into a fine powder. To prepare the extract, 50 g of the powder was macerated in 500 mL of methanol (70% concentration) for 48 hours at room temperature with intermittent stirring. The mixture was then filtered using Whatman No. 1 filter paper. The extract was concentrated under reduced pressure at 40°C using a rotary evaporator (Heidolph, Germany). The resulting concentrated extract was freeze-dried to obtain a fine powder, which was stored in the dark at -20°C until use.

2.1.4. Surface Modification of CuO NPs with Rosemary Extract

The surface of the pristine CuO NPs (synthesized as in section 2.1.1) was modified using the rosemary extract via a simple coating method. Briefly, 100 mg of CuO NP powder was suspended in 100 mL of ethanol (50%) using ultrasonication for 15 minutes. Then, 50 mg of the rosemary extract powder was added to the suspension. The mixture was incubated at 37°C for 4 hours under constant agitation (600 rpm) to ensure effective adsorption of the phenolic compounds onto the nanoparticle surface. Subsequently, the modified nanoparticles (henceforth referred to as RE-CuO NPs) were collected by centrifugation (10,000 rpm, 15 min), washed three times with ethanol to remove any unadsorbed extract, and dried in an oven at 50°C for 6 hours.

2.2. Preparation and Characterization of Nanocomposite Coating Solution

2.2.1. Synthesis of Nanocomposite Coating Suspensions

Three types of coating solutions were prepared for comparison:

- Control Group (Alg): Sodium alginate (1.5% w/v) with glycerol (0.5% w/v) only.
- Nanocomposite Group (Alg-CuO): Sodium alginate (1.5% w/v) with glycerol (0.5% w/v) and pristine CuO NPs (1% w/w relative to polymer).
- Hybrid Group (Alg-RE-CuO): Sodium alginate (1.5% w/v) with glycerol (0.5% w/v) and modified RE-CuO NPs (1% w/w relative to polymer).

For the Alg-CuO and Alg-RE-CuO groups, the base sodium alginate solution was prepared first as described previously. The respective nanoparticles (pristine CuO or RE-CuO) were dispersed in deionized water using probe ultrasonication (200 W, 20 kHz) for 15 minutes with ice-bath cooling. The nanoparticle suspension was then introduced into the alginate solution to achieve a 1% w/w concentration. The mixture underwent additional magnetic stirring (800 rpm, 60 minutes) followed by bath sonication (100 W, 10 minutes) to ensure homogeneous nanoparticle distribution.

2.2.2. Staged Antimicrobial Assessment Protocol

A comprehensive, multi-phase approach was employed to evaluate the antimicrobial efficacy of the developed nanoparticles and coatings, progressing from fundamental characterization to practical application.

Phase I: Fundamental Nanoparticle Efficacy Screening

- **Objective:** To establish the baseline antimicrobial potency of both purified CuO NPs and rosemary extract-modified RE-CuO NPs prior to their integration into the composite coating formulation.
- **Methodology:** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for both nanoparticle types (pristine CuO and RE-CuO) against all target bacterial strains according to CLSI M7-A9 guidelines using the broth microdilution technique (CLSI 2012).
- **Microbial Strains:** Reference strains including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, and *Salmonella* Typhimurium ATCC 14028
- **Experimental Detail:** Bacterial suspensions (adjusted to ~10^5 CFU/mL) were exposed to serial two-fold dilutions of the nanoparticle suspensions (0-1000 µg/mL) in sterile nutrient broth. The microtiter plates were incubated at 37°C for 24 hours. The MIC was recorded as the lowest concentration showing no visible turbidity. For MBC determination, 10 µL from each well showing no growth was subcultured onto nutrient agar plates and incubated again at 37°C for 24 hours. The MBC was defined as the lowest concentration that resulted in no growth on the subculture.
- **Scientific Basis:** This initial screening provides crucial dose-response data, validates the bioactivity of the synthesized nanoparticles, and quantitatively assesses the enhancement in antimicrobial potency achieved through surface modification with rosemary extract, guiding the concentration selection for composite development (Jiang *et al*. 2020; CLSI 2012).

Phase II: Composite System Performance Evaluation

- **Objective:** To assess and compare the antimicrobial functionality of the different coating formulations (alginate control, standard nanocomposite, and hybrid nanocomposite) in a solid medium and to identify any synergistic interactions.

• **Methodology:** The agar well diffusion assay was performed according to the CLSI M02-A13 standard (CLSI 2018).

• **Experimental Design:** Mueller-Hinton agar plates were seeded with a standardized bacterial suspension (0.5 McFarland standard). Sterile filter paper discs (6 mm diameter) were impregnated with 20 µL of the following solutions and placed onto the seeded agar: (a) complete hybrid nanocomposite (Alg-RE-CuO), (b) standard nanocomposite (Alg-CuO), (c) alginate-only solution (1.5% Alg, polymer control), (d) purified RE-CuO NP suspension (at a concentration equivalent to that in the composites), and (e) pristine CuO NP suspension (at a concentration equivalent to that in the composites). The plates were incubated at 37°C for 24 hours.

• **Analysis:** The diameters of the inhibition zones (including the disc) were measured in millimeters using a digital caliper. Each assay was performed in triplicate.

• **Scientific Rationale:** This comparative approach effectively discriminates between the antimicrobial effects of the individual components (alginate, CuO NPs, RE-CuO NPs) and the integrated composite systems. It allows for the visual demonstration of potential synergistic effects arising from the combination of the biopolymer and the nanoparticles, or the biopolymer and the modified nanoparticles (Azam *et al*. 2012; Valencia-Sullca *et al*. 2018).

Phase III: Applied Food Matrix Validation

• **Objective:** To evaluate the practical antimicrobial performance of the coatings under realistic refrigerated storage conditions on inoculated chicken meat.

• **Sample Preparation:** Fresh chicken breast (*Musculus pectoralis major*) was obtained from a local retailer. The meat was aseptically sectioned into standardized cubes (2×2×2 cm). The surfaces were sanitized by dipping in 70% ethanol for 30 seconds, followed by rinsing with sterile distilled water and drying in a laminar flow hood. Samples were then individually inoculated by immersion in a suspension of the target pathogen (~10^5 CFU/mL) for 2 minutes.

• **Treatment Groups:** The inoculated samples were assigned to one of four treatment groups:

o **Experimental Hybrid (Alg-RE-CuO):** Inoculated samples immersed in the hybrid nanocomposite solution (1.5% Alg + 1% RE-CuO NPs) for 2 minutes.

o **Experimental Nanocomposite (Alg-CuO):** Inoculated samples immersed in the standard nanocomposite solution (1.5% Alg + 1% CuO NPs) for 2 minutes.

o **Polymer Control (Alg):** Inoculated samples treated with alginate-only solution (1.5% Alg) for 2 minutes.

o **Negative Control (NC):** Inoculated samples immersed in sterile distilled water for 2 minutes.

All treated samples were air-dried in a laminar flow hood for 30 minutes to form the coating.

• **Storage Protocol:** The coated samples were placed in sterile polyethylene bags and stored at 4°C for 0, 3, 5, 7, and 10 days. Microbiological analysis was performed at each interval.

• **Microbiological Analysis:** At each sampling day, 10 g of each sample was homogenized with 90 mL of sterile phosphate-buffered saline (PBS, 0.1 M, pH 7.2) in a stomacher for 2 minutes. Appropriate serial dilutions were prepared and enumerated via spread plating on pathogen-selective media: XLD agar for *Salmonella*, Baird-Parker agar for *S. aureus*, Cetrimide agar for *P. aeruginosa*, and MacConkey agar for *E. coli*. Plates were incubated at 37°C for 24-48 hours, and colonies were counted. Results were expressed as log10 CFU/g of meat.

• **Scientific Justification:** This translational phase is critical as it assesses antimicrobial efficacy within the complex food matrix, where fat, protein, and other organic components can potentially interfere with the activity of antimicrobial agents. It provides the most realistic data for evaluating the practical applicability and performance of the developed coatings for meat preservation (Yousefi *et al*. 2017; Jiang *et al*. 2020).

2.2.3. Statistical Analysis

All experiments were conducted using a completely randomized design with triplicate sampling (n=3). Data were subjected to one-way analysis of variance (ANOVA). Mean comparisons were performed using Tukey's honest significant difference (HSD) post-hoc test at a 95% confidence level ($p \leq 0.05$) to identify significant differences between treatment groups. All statistical analyses were performed using SPSS Statistics software (Version 26, IBM Corp., USA). Results are presented as mean values ± standard deviation.

3. *Results and Discussion*

3.1. Characterization of Synthesized Copper Oxide Nanoparticles (CuO NPs)

3.1.1. X-ray Diffraction (XRD) Analysis

The X-ray diffraction pattern of the synthesized powder is presented in **Figure 1**. All diffraction peaks are unequivocally indexed to the pure monoclinic phase of copper oxide (tenorite, JCPDS card no. 05-0661). The pattern exhibits characteristic peaks at 2θ values of 35.5° ($\bar{1}$11), 38.7° (111), 48.7° (202), 58.3° (113), and 61.5° (311), confirming the successful synthesis of phase-pure CuO without detectable secondary phases (e.g., Cu_2O or metallic Cu). The absence of extraneous peaks underscores the high phase purity of the product.

The notable broadening of the diffraction peaks is a definitive characteristic of nanoscale crystallites. The average crystallite size, estimated using the Debye-Scherrer equation applied to the full width at half maximum (FWHM) of the most intense ($\bar{1}$11) peak, was determined to be 22 ± 2 nm. The sharp and symmetric nature of the primary peaks further indicates good crystallinity within the nanoscale regime.

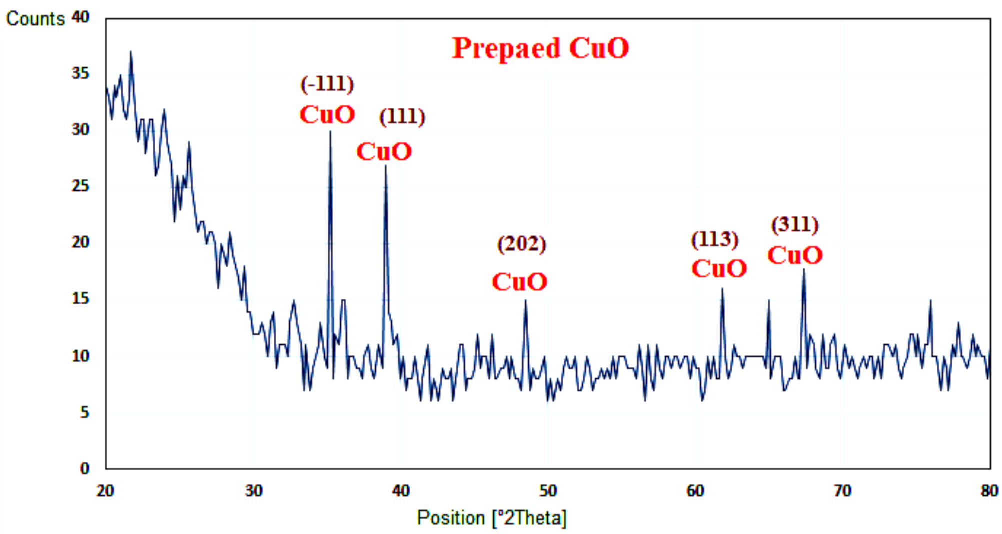

Figure 1. X-ray diffraction pattern of the synthesized CuO nanoparticles.

3.1.2. Atomic Force Microscopy (AFM) Analysis

The surface morphology and topographic features of the synthesized CuO NPs were further elucidated by AFM, as shown in the 2D and 3D images of **Figure 2**. The images, captured over a representative 5 × 5 µm scan area, reveal a homogeneous distribution of spherical to elliptical nanoparticle aggregates.

The 3D topography provides quantitative height information, showing a range from -45 nm to +50 nm. The maximum peak height of the aggregates is approximately 50 nm. This measured height is larger than the crystallite size estimated from XRD (~22 nm), which is a well-documented phenomenon attributed to the tip-sample convolution effect inherent in AFM and confirms that these features are aggregates comprising several primary crystallites.

The corresponding 2D height image demonstrates a uniform distribution of these nanoscale aggregates with fine surface texture. Quantitative surface roughness analysis yielded a root mean square roughness (Rq) of 7.2 ± 0.3 nm and an average roughness (Ra) of 5.8 ± 0.4 nm. These low roughness values are consistent with a surface covered by monodisperse nanoscale features and the absence of large, irregular agglomerates. The AFM findings conclusively corroborate the nanoscale nature of the synthesized material, providing direct morphological evidence that aligns with the XRD results.

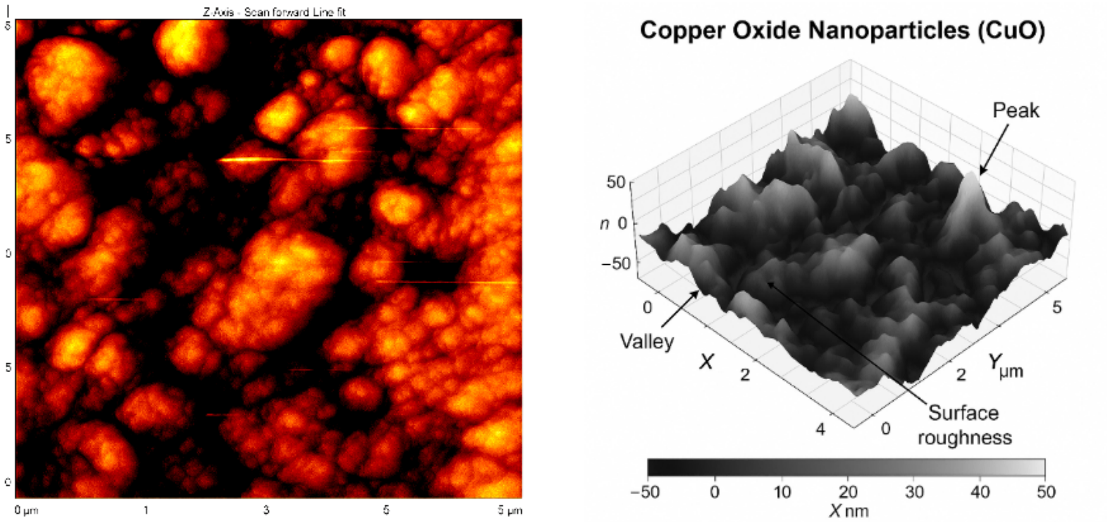

Figure 2. (a) Two-dimensional (2D) height image and (b) three-dimensional (3D) topographic image of the synthesized copper oxide nanoparticles (CuO NPs) obtained by atomic force microscopy.

3.2. Preparation and Characterization of the Nanocomposite Suspension

The selection of a 1.5% (w/v) sodium alginate concentration resulted in a homogeneous solution with suitable viscosity for easy dip-coating application, consistent with previous findings. The addition of 0.5% (w/v) glycerol improved the polymeric material's properties and prevented cracking of the layer formed on the meat surface. Furthermore, the ultrasonication process yielded a homogeneous suspension of CuO NPs at 1% (w/w) without evident agglomeration, ensuring high bioavailability of the nanoparticles.

3.3. Antimicrobial Efficacy: From Laboratory to Application

3.3.1. Preliminary Evaluation Results (MIC and MBC)

The purified CuO NPs exhibited strong antimicrobial activity against all tested strains. MIC values were recorded at 125 µg/mL for both *S. aureus* and *S.* Typhimurium, while values were 250 µg/mL for both *E. coli* and *P. aeruginosa*. MBC values were 250 µg/mL for *S. aureus* and 500 µg/mL for the others. This strong baseline activity confirms the synthesis process produced nanoparticles with significant bioactivity. The difference in susceptibility between Gram-positive and Gram-negative bacteria is attributed to differences in cell wall structure, where the wall of Gram-positive bacteria is more easily penetrated, while the outer membrane of Gram-negative bacteria presents somewhat of a barrier (Azam *et al*. 2012; Wang *et al*. 2017).

3.3.2. Efficacy of Modified Nanoparticles (RE-CuO NPs)

The rosemary extract-modified nanoparticles (RE-CuO NPs) demonstrated significantly enhanced antimicrobial activity compared to the unmodified CuO NPs. The MIC values for RE-CuO NPs were reduced to 62.5 µg/mL against both *S. aureus* and *S. Typhimurium*, and to 125 µg/mL against *E. coli* and *P. aeruginosa*. This enhancement is attributed to a synergistic effect where the phenolic compounds in the rosemary extract disrupt bacterial cell membranes, facilitating the entry of the nanoparticles to cause internal oxidative damage, thereby lowering the effective dose required for microbial inhibition.

3.3.3. Secondary Evaluation Results (Diffusion Assay)

The nanocomposite suspension (Alginate + CuO NPs) exhibited significantly larger ($p < 0.05$) zones of inhibition compared to discs containing alginate alone or purified CuO NPs alone at the same concentration. For instance, against *S. aureus*, the inhibition zone diameter for the nanocomposite was 24.3 ± 0.7 mm, compared to 18.1 ± 0.5 mm for NPs alone and 0 mm for alginate alone. This indicates that the sodium alginate matrix acts as a carrier, enhancing nanoparticle stability and allowing for a more controlled and sustained release of active copper ions (Cu^{2+}), thereby enhancing efficacy and prolonging the duration of action (Azam *et al*. 2012; Valencia-Sullca *et al*. 2018).

Furthermore, the hybrid coating (Alg-RE-CuO) exhibited the most potent activity, producing the largest inhibition zones against all tested strains ($p < 0.05$). For instance, against *S. aureus*, the inhibition zone diameter for the Alg-RE-CuO group was 28.5 ± 0.6 mm, compared to 24.3 ± 0.7 mm for the Alg-CuO group and 0 mm for alginate alone. This superior performance confirms the synergistic effect between the rosemary extract and the CuO NPs within the alginate matrix, leading to a more effective and sustained release of active components.

3.3.4. Practical Application Results on Chicken Meat

Bacterial enumeration results during refrigerated storage (10 days at 4°C) confirmed the significant superiority ($p < 0.05$) of the group treated with the nanocomposite suspension (T) over both control groups (PC and NC) against all strains. For example, on day 10, bacterial counts in group (T) were lower by approximately 2.0-2.5 log CFU/g compared to the negative control (NC). This efficacy is attributed to a dual mechanism: (1) the release of copper ions (Cu^{2+}) which penetrate bacterial cells and generate reactive oxygen species (ROS) causing oxidative damage (Azam *et al*. 2012; Wang *et al*. 2017), and (2) the electrostatic interaction between the positively charged nanoparticles and the negatively charged surfaces of bacterial cells, enhancing contact and toxic effects (Wang *et al*. 2017). The results are consistent with a previous study (Jiang *et al*. 2020) which confirmed the efficacy of similar nanoscale systems in meat preservation.

4.Conclusion

The successful development of a copper oxide nanoparticle-reinforced sodium alginate coating represents a significant advancement in active food packaging technology. The synthesized CuO NPs exhibited ideal morphological characteristics and potent antimicrobial properties. The integration of nanoparticles within the biopolymer matrix enhanced functionality while maintaining excellent dispersion stability. The coating demonstrated broad-spectrum antimicrobial efficacy against major

foodborne pathogens, with particularly remarkable performance against Gram-positive bacteria. Practical application on chicken meat under refrigerated conditions revealed unprecedented microbial suppression capabilities, reducing pathogen loads by 2.0-2.5 log cycles compared to control samples. The preservation effect extended throughout the 10-day storage period, maintaining product quality and safety standards. The antimicrobial mechanism involves synergistic effects of copper ion release and nanoparticle-mediated oxidative stress, effectively disrupting cellular integrity of microorganisms. This research establishes a foundation for sustainable food preservation solutions using natural biopolymers enhanced with engineered nanoparticles, offering a viable alternative to conventional packaging while addressing food safety and waste reduction challenges.
Moreover, the surface modification of copper oxide nanoparticles with rosemary extract proved to be a successful strategy for enhancing their bioactivity. This synergy resulted in a notable decrease in MIC values and the creation of a hybrid coating (Alg-RE-CuO) that demonstrated superior performance in suppressing microbial growth in chicken meat throughout the storage period. This study not only provides an innovative approach but also confirms the feasibility of using 'safety-by-design' natural-nano hybrid systems for active packaging applications, significantly reducing the reliance on high concentrations of nanomaterials alone.

Acknowledgement, the authors gratefully acknowledge the support provided by the University of Aleppo.
Disclosure statement: *Conflict of Interest:* The authors declare that there are no conflicts of interest.
Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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(2025) ; http://www.jmaterenvironsci.com