

المؤتمر العلمي الدولي السابع عشر للعلوم الصرفة والتطبيقية والتكنولوجية

**XVII. International Scientific Congress of Pure, Applied
and Technological Sciences**

**XVII. Uluslararası Fen, Uygulamalı ve Teknolojik
Araştırmalar Kongresi**



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PREFACE

XVII. International Scientific Congress of Pure, Applied and Technological Sciences was organized by Harran University in collaboration with Rimar Academy. The primary objective of this event was to compile and disseminate valuable scientific knowledge and make a meaningful contribution to the future.

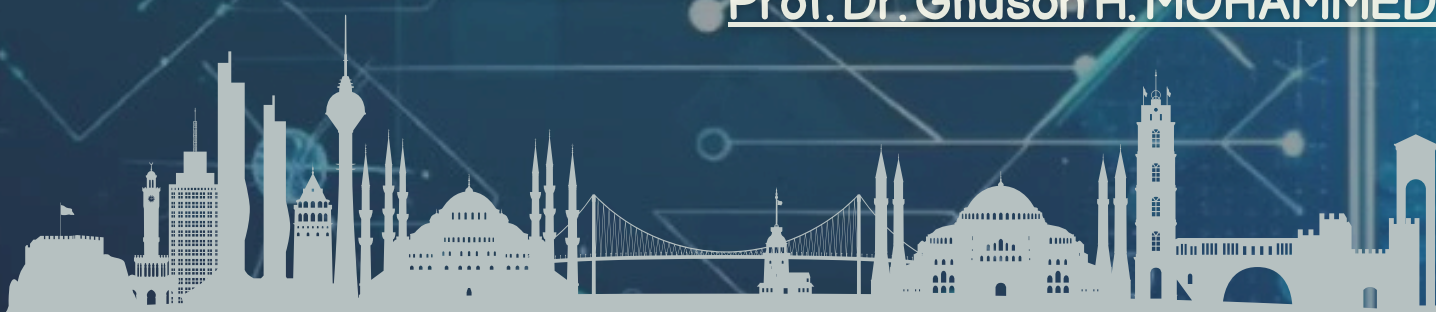
A substantial number of researchers from both local and international backgrounds demonstrated their interest in this conference. The scientific committee meticulously reviewed the submissions and ultimately accepted a select group of applicants—35 in total—of whom 30 were approved by the scientific committee.

The core of this conference was the presentation of 15 full research papers, while the remaining articles and research findings are set to be featured in for the coming issues of the MINAR Journal.

I would like to extend my sincere appreciation to all the contributors and scholars who played an essential role in making this conference a resounding success.

Your dedication and valuable contributions are deeply respected and acknowledged.

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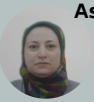
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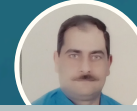
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Evaluation of *Bacillus subtilis* as a Biocontrol Agent Against *Fusarium Oxysporum* and Its Influence on Growth and Defense Gene Expression in *Triticum Aestivum* L

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Muna Mohammed Khayri ²

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Abstract:

Fusarium oxysporum (Fo), a soil-borne phytopathogen, is one of the most damaging fungal infections of wheat (*Triticum aestivum* L.), one of the most important cereal crops worldwide. This research used *Bacillus subtilis* as a plant growth-promoting rhizobacterium to boost wheat growth and fungus resistance. Rhizobacteria from wheat rhizosphere soil were newly isolated and morphologically and biochemically described. We tested the chosen bacterial isolate's *Fusarium oxysporum* antagonistic activity using dual culture. The *in vitro* experiments demonstrated that bacterial inoculation promoted plant growth and defense against fungal pathogens in wheat plants under the different treatments (control plants, bacterial treatment, fungal infection, and combined) [1]. Moreover, Quantitative PCR was employed to determine the expression profile of PR1. The findings indicated that *Bacillus subtilis* showed high antifungal activity against *Fusarium oxysporum* and enhanced wheat growth parameters by a large margin, including plant height, root length, and biomass. Compared with plants infected with only the pathogen, those treated with the bacterial isolate Was exhibit reduced disease severity. Quantitative analysis of PR1 gene expression by qPCR showed increased levels for all three treatments (with bacterial and fungal pathogens) but significantly higher for those challenged with the bacterial treatment. These results imply that *Bacillus subtilis* is a beneficial microorganism for growth promotion and activation of plant defense responses. Hence, application of beneficial rhizobacteria might be considered as sustainable and environmentally friendly route for managing the fungal diseases and enhancing productivity of wheat.

Keywords: Biological Control, *Bacillus Subtilis*, *Fusarium Oxysporum*, Pr1 Gene, Wheat.



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Introduction

Wheat (*Triticum aestivum* L.) is a key cereal crop and source of calories and protein worldwide. Wheat is a basic food crop that helps secure global food security, especially in underdeveloped countries and dry regions. Many Middle Eastern nations, like Iraq, rely on wheat farming for sustenance. Fungal infections are one of the biggest biotic stressors that reduce wheat production and quality. *Fusarium oxysporum*, a major soil-borne fungal pathogen, infects numerous agricultural plants, including wheat. This fungus spreads from root to vascular tissue, blocking water and nutrient transport in the plant. This pathogen causes root rot, wilting and chlorosis, juvenile plants show reduced growth, productivity is adversely affected. This pathogen is particularly difficult to control due to its ability to persist in the environment (in soil and plant residues). Fungal diseases have typically been managed in crops with chemical fungicides. While these compounds effectively decrease the disease incidence, their over-usage has been reported to carry an environmental risk as well as accelerate pathogen resistance and expose human health. Thus, more and more focus has been on generation of sustainable and eco-friendly disease management options. A potential approach includes beneficial microorganisms, especially Plant Growth-Promoting Rhizobacteria (PGPR) [8,14,15]. They colonize the roots with plants; as result, they promote plant growth via different mechanism like phytohormones production and nutrient solubilization through the process of biological nitrogen fixation [5,21,26]. Moreover, PGPR could also suppress plant pathogens by producing antimicrobial compounds, competing for nutrients and inducing plant defense responses [3,6,9]. Among PGPR species, *Bacillus subtilis* has gained wide interest owing to its potential to synthesize different bioactive metabolites and antimicrobial agents [2,16,38]. This Gram-positive, spore-forming bacterium is widely distributed in soil ecosystems and has been shown as effective biocontrol of several plant pathogens [7,14,31]. In addition to its antagonistic actions, *B. subtilis* induces systemic resistance in plants [2,29,36]. Plant defense responses usually activate defense-related genes [18,39,40]. PR1, one of the most researched immune response genes, is a typical marker for plant systemic acquired resistance to pathogens [19,36]. Pathogens or helpful microbes may activate plant defense mechanisms and express this gene [9,19]. Recently, molecular biology technologies have developed, notably qPCR, which can reliably study several plant gene expression patterns [20]. This strategy elucidates the molecular basis of plant-microbe interactions as well as plant defense responses [18,39]. Results indicate the potential of *Bacillus subtilis* as a plant growth-promoting rhizobacterium to enhance wheat development and plant defense responses against *Fusarium oxysporum* infection. This study specifically aims to isolate and characterize rhizobacteria from wheat rhizosphere soil, test their antagonistic activity against the fungal pathogen, evaluate the impact on growth parameters of wheat under greenhouse condition and examine expression of defense related PR1 gene via qPCR.

Materials and Methods

1. Study Design:

This experiment examined how helpful rhizobacteria improve wheat growth and fungus resistance. Experimental workflow has four main processes: (i) Isolation and characterization of wheat rhizobacteria, (ii) fungal pathogen preparation, (iii) greenhouse assessment of wheat growth against biological treatments, and (iv) quantitative real-time polymerase chain reaction analysis of plant defense gene expression.

2. Collection of Soil Samples:

Fields cultivated for wheat had soil samples taken from the rhizosphere. In order to gather soil microorganisms associated with the rhizosphere, sterile instruments were used to carefully gather soil that was strongly adhered to the wheat roots. Septic sample bags were filled with about 50 g of dirt and brought to the lab in a refrigerated environment. Isolation of microbes required processing of samples kept at 4°C for no more than 24 hours.

3. Isolation of Rhizobacteria:

Shaking a random 10-gram sample of rhizosphere soil with 90 mL of sterile distilled water homogenized it. We diluted the sample up to 10^{-6} in sterile saline solution. Spread 100 μ L of each dilution onto Nutrient Agar plates using a sterile spreader. At 28°C, plates incubated for 24–48 h. Purifying 6 separate bacterial colonies by streaking them numerous times onto fresh Nutrient Agar plates yielded pure cultures based on morphology, color, and form [8,14,24].

4. Identification of Bacterial Isolates:

Morphological and biochemical tests were conducted to characterize the purified bacterial isolate. The colony morphologies, such as form, pigmentation, size and texture were recorded. The Gram reaction and cellular morphology viewed by light microscope were examined by performing the Gram staining. Standard biochemical tests such as catalase test, oxidase test and starch hydrolysis were performed to aid identification of the isolates. On the basis of these characteristics, the bacterial isolate employed for further studies was identified as *Bacillus subtilis* [2,14,17].

5. Preparation of the Fungal Pathogen:

Fungarium oxysporum was cultivated on Potato Dextrose Agar (PDA) plates at 25–28°C for 5–7 days until mycelial growth was adequate. We sub cultured fungal cultures on PDA slants to sustain vitality for experiments [4,11].

6. In Vitro Antagonistic Activity Assay:

Dual culture tested the bacterial isolate's fungal pathogen antagonistic capabilities. The center of PDA plate included a 5 mm agar disc from actively growing fungal mycelium. After streaking *Bacillus subtilis* on PDA plates 2-3 cm from the fungal disk, all plates incubated for 5 days at 28 °C. Distance between bacterial and fungal mycelia growth fronts measured antifungal activity. Determined fungus inhibitory growth proportion [3,6,16].

7. Plant Material and Seed Preparation:

Wheat (*Triticum aestivum* L.) seeds were purchased from a local farm store. Seed was surface sterilized through 1-minute immersion in 70% ethanol followed by a 3-minute treatment with a sodium hypochlorite (1%, v/v) solution. After that, the seeds were dried under sterile circumstances and rinsed three times with distilled water to remove any disinfectant residues. Only then were they ready to be planted [1,15,26].

8. Greenhouse Experiment:

Planting wheat seedlings in sterilized plastic containers. Each container included three seeds and one seedling following germination. Grown in greenhouses with constant watering. Establishing four experimental therapies:

1. Control treatment: plants grown without bacterial or fungal inoculation.
2. Bacterial treatment: plants inoculated with the selected rhizobacterial isolate (*Bacillus subtilis*) using 10 mL bacterial suspension adjusted to approximately 10^8 CFU/mL [2,7].
3. Pathogen treatment: plants inoculated with the fungal pathogen *Fusarium oxysporum* only using actively growing fungal culture added to the rhizosphere soil [11].
4. Bacteria + pathogen treatment: plants treated with *Bacillus subtilis* followed by fungal infection seven days later [2,9].

9. Bacterial Inoculation of Plants:

Nutrient broth was inoculated with *Bacillus subtilis* and incubated for 24 hours to prepare the bacterial suspension. The culture was centrifuged down and resuspended in sterile saline solution to yield approximately 10^8 CFU/mL. About 10 mL of the bacterial suspension was added at the vicinity of each root zone [2,7,27].

10. Fungal Inoculation:

The fungal pathogen *Fusarium oxysporum* was introduced by adding a small volume of actively growing fungal culture in the soil around the plant root zone. To assess the protective effect of rhizobacteria, inoculation was performed seven days post bacterial treatment [9,11,31].

11. Measurement of Plant Growth Parameters:

Drying plants from pots during this experimental growth phase assessed plant height, root length, fresh weight, and dry weight. Recording disease symptoms helped determine fungal infection severity.

12. RNA Extraction:

Following the manufacturer's instructions, a commercial plant RNA extraction kit retrieved total RNA from wheat leaf tissues. We powdered 100 mg of leaf tissue in liquid nitrogen to isolate RNA. Spectrophotometry measured RNA content and purity [18,20].

13. cDNA Synthesis:

Follow the manufacturer-provided methodology to create complementary DNA (cDNA) from purified RNA using a reverse transcription kit. We followed the manufacturer's procedure to conduct a reverse transcription reaction using total RNA (about 1 µg) as our template. [20].

14. Quantitative Real-Time PCR (qPCR) Analysis:

Quantitative real-time RT-PCR analyzed gene expressions. Primers for the wheat defense gene PR1 amplified the target sequence's cDNA. After standard cycling, the real-time PCR equipment analyzed the target utilizing cDNA template, gene-specific primers, SYBR green master mix, and nuclease-free water for an amplified detection run. We calculated differential expression levels using comparative Ct method ($2^{-\Delta\Delta Ct}$) [19,20].

15. Statistical Analysis:

Each experiment included three runs. We provided data as means $\pm$ SD. We compared treatments using a one-way analysis of variance (ANOVA) and separated means using Tukey's post hoc test at a significance level of $P < 0.05$.

Results

1. Antagonistic Activity of *Bacillus subtilis* Against *Fusarium oxysporum*:

Perform a dual culture experiment between the fungal pathogen and the bacterial isolate following the procedures described in the Materials and Method section and determine if there are any antagonistic activities. There was a clear zone of inhibition after the incubation of the bacterial colony and the fungal mycelium. In the case of the control plates – *Fusarium oxysporum* only, it extended over the entire surface area of the PDA visited. The growth suppression of fungi was demonstrated on the contrast media plates inoculated with *Bacillus subtilis*. The diameter of the fungal colony significantly reduced in control treatment (Table 1).

Table 1. Inhibition of *Fusarium oxysporum* growth in dual culture assay

Treatment	Fungal Colony Diameter (mm)	Inhibition (%)
Fungus only (Control)	85 $\pm$ 2.1	0
Bacteria + Fungus	32 $\pm$ 1.8	62.3

Values with various capital letters vary considerably ($p < 0.05$).

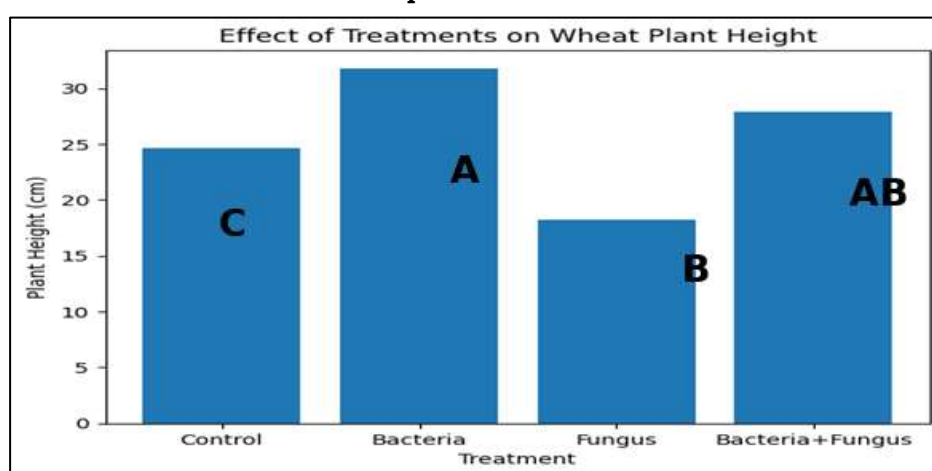
2. Effect of Bacterial Inoculation on Wheat Growth:

Measurements included plant height, root length, and fresh and dry weight. *B. subtilis*-treated plants grew better. Plants infected with fungus only displayed lower growth and disease signs. It was striking that plants stressed with *Bacillus subtilis* before fungal infection grew better than fungus alone, indicating that the isolate protects plants. Statistical analysis using ANOVA revealed significant differences across treatments ($p < 0.05$) (see Table 2 and image 1t).

Table 2. Effect of treatments on wheat growth parameters

"Treatment	Plant Height (cm)	Root Length (cm)	Fresh Weight (g)	Dry Weight (g)	P-value
Control	24.6 ± 1.2 C	11.4 ± 0.8 C	2.8 ± 0.3 C	0.82 ± 0.06 C	<0.05
Bacteria (<i>Bacillus subtilis</i>)	31.8 ± 1.5 A	16.3 ± 1.1 A	3.9 ± 0.4 A	1.18 ± 0.09 A	<0.05
Fungus (<i>Fusarium oxysporum</i>)	18.2 ± 1.0 B	7.6 ± 0.5 B	1.7 ± 0.2 B	0.51 ± 0.04 B	<0.05
Bacteria + Fungus	27.9 ± 1.3 AB	13.7 ± 0.9 AB	3.1 ± 0.3 AB	0.96 ± 0.07 AB	<0.05"

"Values are mean ± SD (n = 3). Different capital letters indicate significant treatment differences at $p < 0.05$ ".

**Figure 1. Effect of treatments on wheat plant height**

3. Disease Severity Assessment:

Disease symptoms were monitored in all treatments throughout the experimental period. Plants infected with *Fusarium oxysporum* exhibited symptoms such as root discoloration, reduced growth, and partial wilting. However, wheat plants treated with *Bacillus subtilis* before pathogen inoculation displayed markedly reduced disease symptoms. The bacterial treatment significantly lowered the severity of fungal infection compared with plants exposed to the pathogen alone Table 3.

Table 3. Disease severity in wheat plants under different treatments

Treatment	Disease Severity (%)
Control	0
Fungus	71
Bacteria + Fungus	29

"Different uppercase letters indicate that treatments means differ significantly ($p < 0.05$)".

4. Defense Gene Expression Analysis:

Quantitative real-time PCR (qPCR) assayed PR1 gene expression. Quantitative PCR using gene-specific primers amplified wheat leaf total RNA. The findings showed that *Bacillus subtilis* and *Fusarium oxysporum* substantially enhanced PR1 gene expression. Plants treated with both bacterial and fungal pathogens had the greatest gene expression, suggesting defensive responses and resistance mechanisms. Table 4 and Figure 2 reveal significant variations in PR1 gene expression across treatments ($p < 0.05$).

Table 4. Relative expression of PR1 defense gene in wheat plants

Treatment	Relative Expression (Fold Change)	P-value
Control	1.0 C	-
Bacteria (<i>Bacillus subtilis</i>)	2.6 BC	<0.05
Fungus (<i>Fusarium oxysporum</i>)	3.4 B	<0.05
Bacteria + Fungus	5.8 A	<0.05"

"Different uppercase letters indicate significant differences among treatments at $p < 0.05$ ".

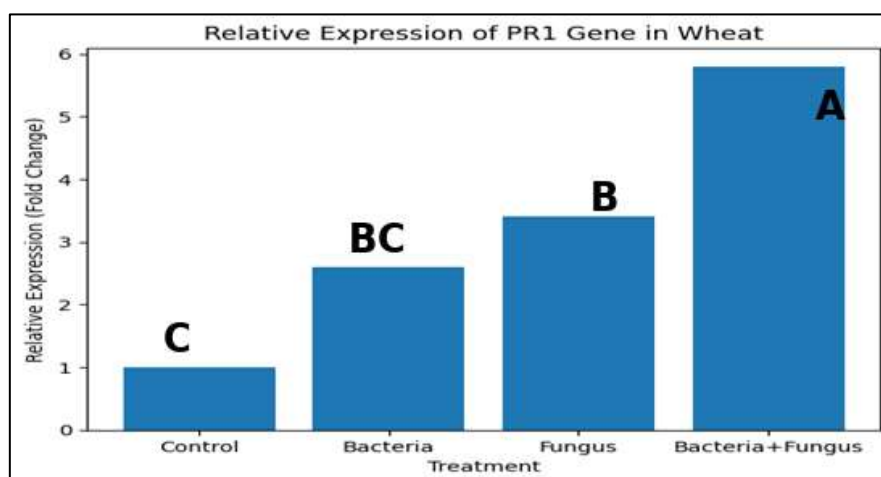


Figure 2: Wheat plant PR1 defensive gene expression relative to other plant types

Discussion

This research shows that *Bacillus subtilis* may boost wheat growth and reduce fungal infestation. The isolate's dual culture test antagonistic activity against *Fusarium oxysporum* shows significant antifungal properties. A prominent inhibition zone between the bacterial colony and the fungal mycelium implies *Bacillus subtilis* generates antifungal compounds that impede fungal development. Several bioactive substances, including lipopeptides, which suppress fungal infections, may explain this effect.

Bacillus species generate antibiotics and hydrolytic enzymes that break down fungal cell walls [10,16]. These metabolites inhibit fungal pathogen growth in the rhizosphere [6,31]. *Bacillus subtilis*' percentage of inhibition demonstrated it might control soil-borne diseases, supporting prior research [3,11,14]. The bacterial therapy boosted greenhouse wheat growth in addition to its antagonistic effects. Inoculated wheat plants had higher height, root length, and biomass than untreated control plants due to *Bacillus subtilis*. Our results support many

studies indicating plant growth-promoting rhizobacteria promote plant development via phytohormone synthesis, nutrient enrichment, and root system development [5,8,15,27]. The current study demonstrated that infection with the plant pathogen, *Fusarium oxysporum* negatively affected wheat growth; consistent with the pathogenic properties of this filamentous fungus [4,11]. This indicates that infection with this pathogen interferes with normal physiological processes in the plant, resulting in stunted growth and clear symptoms of disease. However, wheat plants that were treated with the *Bacillus subtilis* before they became infected with the fungus exhibited enormous improvement in growth versus plants that were just infected with the pathogen. The bacterial isolate indicates protective role with the fungal infection. Similarly, the lower disease severity ratings obtained in response to treatment with both bacteria and fungi provide further evidence for *Bacillus subtilis* acting as a biological control agent. Thus, the bacterial isolate may have inhibited pathogen development through direct antagonism and competition for nutrients and ecological niches such as in the rhizosphere [6,31]. Prior studies have yielded similar observation where *Bacillus* species successfully minimized the severity of soil-borne fungal diseases in diverse crops [2,7,14].

Gene expression analysis of the defense-related gene PR1 showed significant changes for both bacterial treatment and fungal infection at the molecular level. Higher up-regulation of this gene in *Bacillus subtilis*-treated plants indicates activation of defense pathways by the bacterium. It is most likely that the highest level of gene expression in response to dual treatment with both bacteria and fungus indicates a primed plant immune system, thus allowing for a greater breadth in defense action during pathogen attack. Such responses may strengthen plant immunity and improve the plant's ability to respond to pathogen invasion [18,39,40]. Systemic acquired resistance (SAR) is commonly verified through the molecular marker PR1, and its expression is typically elevated when plant defense pathways are activated [19,36]. Beneficial rhizobacteria are known to induce defense signaling pathways superimposed over the mechanisms of resistance in plants [9,29,36]. Consequently, the increased expression of PR1 in this study molecularly proves that *Bacillus subtilis* induces plant defense responses. In conclusion, the results presented in this study underline the significance of beneficial rhizobacteria for enhancing plant health and disease resistance against fungal pathogens. A general trend of using an additive model finds that the various effects of pathogen suppression, plant growth promoting activity, and induction of defense-related genes suggest a combinatorial effect by *Bacillus subtilis* in plant-microbe interactions. The results show that rhizobacteria beneficial could be an environmentally friendly and sustainable alternative for the control of wheat fungal diseases [8,14,15]. Additional detailed characterization of plant-defense responses at the field scale and more molecular characterization of such responses may shed further light on these desirable interactions. In the present study, the promising results were obtained in the greenhouse, and under field conditions, more experiments have to be conducted to design effective strategies to use *Bacillus subtilis* in natural agricultural environment.

Conclusion

Bacillus subtilis shown remarkable potential as a plant growth-promoting rhizobacterium that may boost wheat growth and reduce *Fusarium oxysporum* infection in this investigation. Finally, beneficial rhizobacteria may be an eco-friendly and sustainable alternative to fungal disease management and wheat yield.

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Histopathological and Molecular Effects of Zinc Oxide Nanoparticles on Tracheal Tissue in Mice

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Abstract:

NPs)—a cationic commercial dispersion and a raw nanopowder—on macrophage cell lines (RAW 264.7 and J774A.1) and primary bone marrow-derived macrophages. Physicochemical characterization via Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) revealed that both NP types form stable aggregates ranging from 200 to 250 nm, which undergo significant intracellular dissolution, reducing to fragments as small as 25 nm within endocytic vesicles.

Quantitative viability assays established the LD₂₀ at relatively low concentrations (4–8 µg/mL), indicating high potency compared to other metallic oxides. Comparative analysis between nanoparticulate and ionic zinc (Zn²⁺) suggests that toxicity is largely driven by the released ionic fraction. Comprehensive proteomic profiling identified 53 differentially expressed protein spots, primarily associated with central metabolism, oxidative stress regulation, and cytoskeletal integrity.

The study concludes that ZnO NP toxicity is a multi-faceted process governed by ionic interactions with cellular machinery. Macrophages successfully employ compensatory metabolic and antioxidant strategies up to a specific threshold, beyond which homeostatic failure leads to cell death.

Keywords: ZnO Nanoparticles, Cytotoxicity, Proteomics, Macrophages, Glycolysis, Oxidative Stress, Ionic Dissolution.



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1_Introduction

For this part, two types of ZnO nanoparticles (NPs) were studied: firstly, a commercial dispersion containing ZnO NPs suspended at 50% (w/v) in an aqueous solution containing a cationic dispersing agent (3-aminopropyl triethoxysilane), which will be referred to hereafter as "cationic ZnO"; and secondly, NPs in the form of a nanopowder with an average diameter of approximately 50 nm. In the first case, the dispersion was characterized solely by DLS and electron microscopy, and then used as is, diluted to working concentrations in the culture medium. In the case of the nanopowder, it was necessary to optimize the dispersion conditions of the suspended NPs, as in the case of copper.

1.1. Optimization of NP dispersion and characterization by DLS:

Given the optimizations already performed on copper nanoparticles (NPs), and in particular CuO, we hypothesized that the behavior of ZnO would be similar. Suspensions were therefore prepared at a high concentration (5% (w/v), or 50 mg/mL). Several stabilizing agents were tested, but simple sonication in water provided the best dispersion. As with copper, the dispersion of the nanoparticles (NPs) under different conditions was also analyzed by DLS after dilution to 10 µg/mL of NP, both in water and in serum culture medium, since the latter appears to improve the NP size distribution. Finally, the stability of the tested dispersions was evaluated after 24 hours and then over a period of several weeks. The diameters measured by DLS for the nanopowder dispersed in the presence of the different coating agents, as well as for the commercial dispersion, are presented in Table 1.

Suspension / Dispersing Agent	Diameter in		Stability after 24h
	H ₂ O (nm)	+ SVF 1% (nm)	
ZnO cationique 50%	146	217	No aggregation
ZnO powder 5%	Sterile H ₂ O	247	No aggregation
	PVP40 1%	252	Partially settled
	BSA 1 mg/mL	1815	Completely sedimented
	Gelatin 1 mg/mL	419	Completely sedimented
	Dextran 2%	310	No aggregation

As with copper nanopowders, none of the different dispersing agents allowed for the production of zinc nanoparticles with a diameter smaller than 100 nm: the nanoparticles generally formed aggregates with a minimum diameter of approximately 250 nm. This factor must therefore be taken into account when analyzing the study results. It can also be noted that, as before, the presence of SVF in the culture medium improves dispersion, with the exception of nanoparticles without a stabilizing agent, for which no significant size difference was observed between measurements taken in water and in serum medium. These results are therefore consistent with those of the copper study and with data from the literature.

Table 1: Maximum diameters of ZnO NPs measured by DLS in water or culture medium, in the presence of different dispersing agents.

Furthermore, Table 1 also shows that, in the case of zinc, sonication in water seems sufficient to obtain satisfactory dispersion, since the coating agents tested do not reduce the minimum aggregate size, although PVP40 gives results close to those obtained in water alone so it was not chosen for subsequent work. It therefore appears that simple sonication of the zinc NPs in water alone is the best and most stable dispersion method for these particles. Furthermore, the absence of a stabilizer will allow for the evaluation of the actual effects of the zinc NPs themselves. Regarding the commercial dispersion (cationic ZnO), dilution of the initial suspension for DLS measurements appears to induce the formation of aggregates on the order of 150 nm in diameter in water, and 217 nm in medium with SVF (Table 1 and .

Figure 1). This size increase in culture medium, despite the presence of serum, could not be fully explained. However, it could be related not to the particles themselves, but to the stabilizing agents introduced into the suspension by the supplier, and to their Interactions with the components of the medium and/or serum occur, as this phenomenon is not observed in the case of raw metal nanopowders or metal oxides. However, the dimensions observed for this dispersion remain of the same order of magnitude as those obtained for the nanopowder dispersed in water, and are also stable over time. Furthermore, whether for ZnO/H₂O or cationic ZnO, the dispersion histograms obtained by DLS also show that both types of suspensions are quite homogeneous (narrow distributions), although in the case of the powder, dilution in serum medium induces a slight loss of homogeneity (Figure 1). These histograms also confirm the dimensions of the nanoparticles (NPs) in these suspensions: approximately 150 nm in water and 300 nm in medium with FBS for cationic ZnO, and approximately 200 to 250 nm for the ZnO/H₂O NPs in water and in FBS.

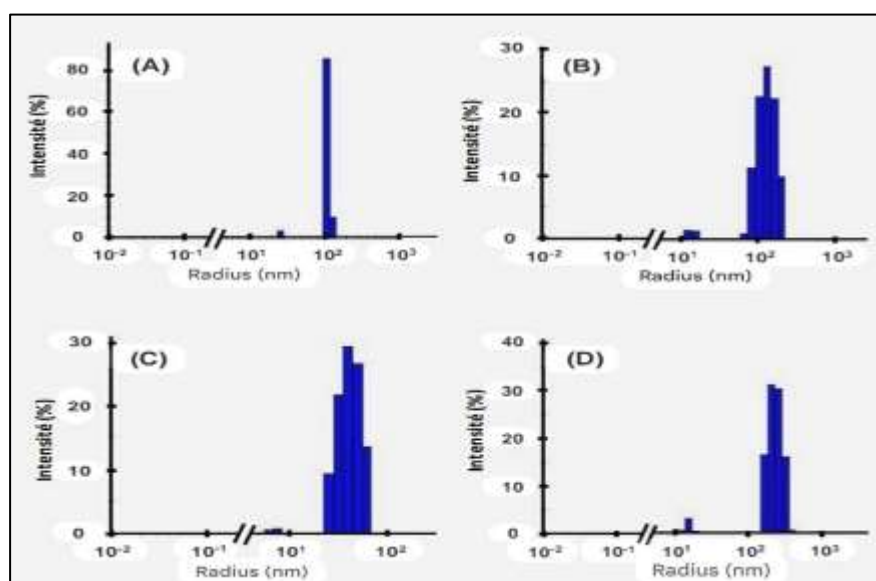


Figure 1: Size dispersion histograms of ZnO NPs obtained by DLS. (A) ZnO/H₂O measured in water; (B) ZnO/H₂O measured in DMEM+SVF 1%; (C) Cationic ZnO measured in water; (D) Cationic ZnO measured in DMEM+SVF 1%.

Finally, regular DLS measurements were performed over a period of several weeks and showed that the ZnO/H₂O suspensions are stable for 6 to 7 weeks (results not shown here). Beyond this period, the particles tend to agglomerate. New suspensions must therefore be prepared and characterized to ensure the reproducibility of the cell exposure conditions to elements of consistently nanometric dimensions.

1.2. Characterization of ZnO Suspensions by Electron Microscopy:

To complement the data obtained by DLS, we focused here on the characterization of NPs after their internalization by cells. These cells were exposed to ZnO/H₂O and cationic ZnO NPs, then fixed with glutaraldehyde and analyzed by transmission electron microscopy (TEM). The results presented are those obtained on the J774A.1 cell line

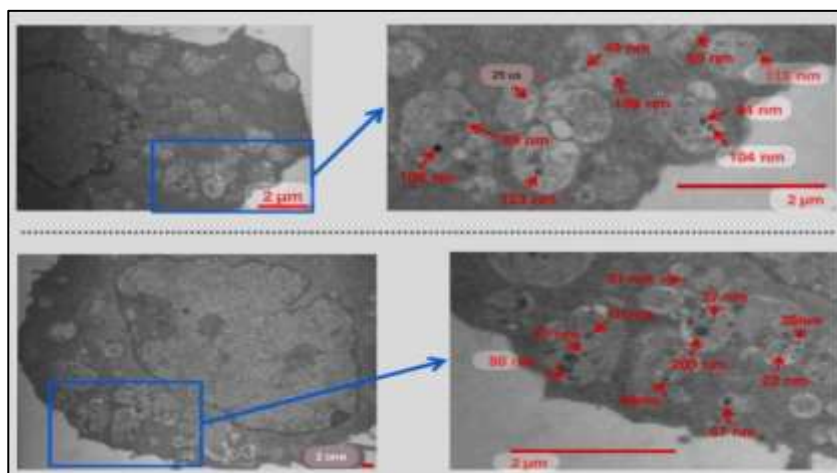


Figure 2: Characterization of cationic ZnO (top) and ZnO/H₂O (bottom) NPs by TEM.

Cells were treated with 4 µg/mL of cationic ZnO or 8 µg/mL of ZnO/H₂O, then fixed, and counterstained with lead citrate and uranyl acetate before being visualized by TEM. The micrographs on the right correspond to enlargements of the areas outlined in blue on the left. Arrows indicate the NPs observable in the intracellular vesicles, along with their size calculated from the corresponding scale bar. As shown in Figure 2, the NPs internalized by the cells are mainly observed in intracellular vesicles as dark-colored, spherical granules. The cationic ZnO and ZnO/H₂O NPs have diameters of up to approximately 125 nm and 200 nm, respectively, which is smaller than their dimensions in suspension.

1.3. Evaluation of the degree of dissolution of ZnO in culture media:

Based on previous observations and the results obtained for copper nanoparticles (NPs), the ionic fraction released into the cell culture medium from the ZnO NPs was also estimated. The NPs were introduced for 24 hours into RPMI medium containing 10% FBS (the culture and exposure conditions of the cell lines used in this study). Since no significant difference was observed in the case of copper between the conditioned and unconditioned media, this measurement was performed only in the unconditioned medium. After removal of the NPs by ultracentrifugation, the Zn²⁺ ions were measured using xylenol orange, another indicator that

forms complexes with certain metal ions, particularly iron (Fe or Fe³), but also zinc (Zn²⁺), thus allowing their detection by colorimetry (Gay, C., et al., 1999).

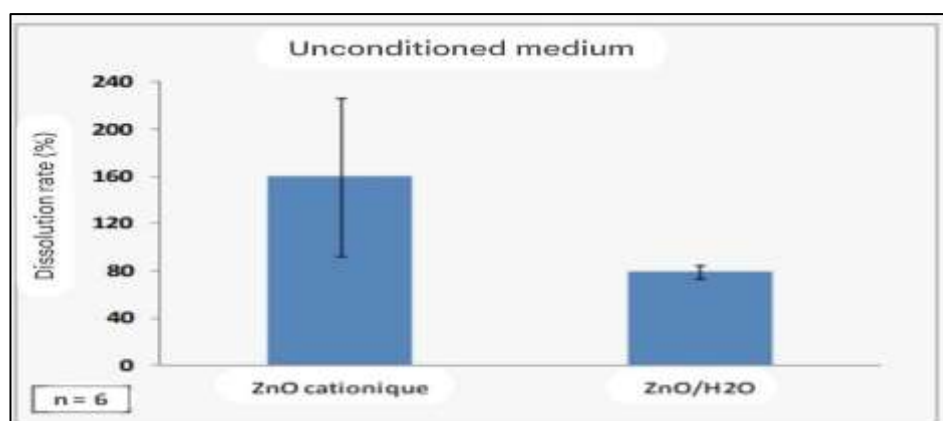


Figure 3: Determination of the ionically dissolved fraction from ZnO NPs in cell culture medium.

After 24 hours of incubation in culture medium (RPMI+SVF 10%), ZnO/H₂O NPs are found to be on average 80% dissolved in ionic form. This high dissolution rate has also been observed by other groups: Hackenberg et al. However, in the work presented here, we used fairly low concentrations (on the order of 8 µg/mL), which could explain the high dissolution rate observed. As with copper, the contribution of this significant ionic fraction could be taken into account when assessing the toxic effects of NPs despite significant NP dissolution at low concentrations, the effects of the ionic fraction remain negligible, as NPs have much lower cytotoxicity than ions alone. The differences observed between the two studies could be partly explained by the fact that the NPs are of different sizes and shapes, as are the viability tests used. Furthermore, TEM images show that NPs remain relatively large inside cells, indicating rapid internalization. However, it seems worthwhile here to also measure the intracellular dissolved fraction.

Regarding cationic ZnO, the results obtained did not allow us to determine the dissolution of the NPs. The observed levels and very high standard deviations could in this case be explained by the presence of cationic dispersing agents introduced by the supplier, which are assumed to be the source of interference with the assay. As no further information could be obtained from the supplier on this subject, information on the dissolution of this type of NP remains unavailable.

2 Materials And Methods

The cytotoxicity of cationic ZnO and ZnO/H₂O nanoparticles, as well as that of Zn ions, was evaluated in both macrophage cell lines after exposing the cells to different concentrations of nanoparticles or a zinc acetate ((CH₃COO)₂Zn) solution. The results of the cell viability tests after 24 hours are shown in Figure 4. These tests allowed us to determine the LD₂₀ for both cell lines to be used for the remainder of the study. This LD₂₀ is 4 µg/mL for cationic ZnO and 8 µg/mL for ZnO/H₂O, and no significant difference in response was observed between the two cell lines (Figure 4). As with Cu and CuO nanoparticles, it is worth noting that these

doses are relatively low compared to those used for other metal oxides, such as TiO_2 (Osman, I.F., et al., 2010; Kim, I-S., et al., 2010). Beyond this dose, the cell death rate increases sharply, particularly with the cationic form, reaching almost 100% at 10 $\mu\text{g/mL}$ in both cell lines, whereas an equivalent dose of the nanopowder form induces 35 to 50% mortality. It can be assumed that this difference in response to the two types of nanoparticles could stem from the cationic coating, which may be toxic in itself or exacerbate the toxicity of the nanoparticles. Indeed, this type of modification can induce changes in the overall surface charge of the nanoparticles. However, an increase in positive charges can promote not only interactions between the nanoparticles and biological constituents, which can alter them, but also the internalization of NPs by cells, or the dissolution of NPs into ions that can in turn penetrate cells and have their own toxicity (Chang, Y.N., et al., 2012).

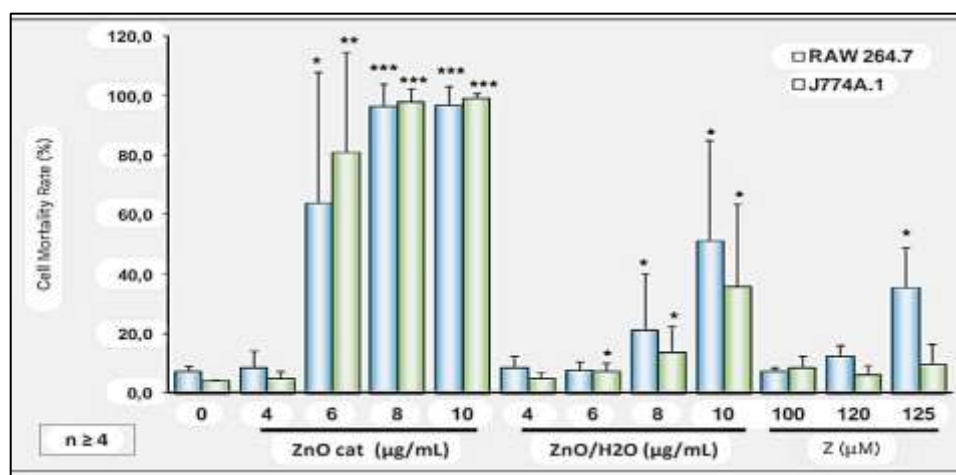


Figure 4: Cell mortality rate of RAW 264.7 and J774A.1 cell lines in the presence of NPs and zinc ions. Cells were exposed for 24 h to different doses of cationic ZnO_2 , $\text{ZnO}/\text{H}_2\text{O}$, and Zn ions.

The mortality rate was then determined by the Trypan Blue exclusion test. Statistical significance: * for $p \leq 0.05$, ** for $p \leq 0.01$, and *** for $p \leq 0.001$ according to the non-parametric Mann-Whitney U test, the same phenomena in cells from human lung carcinoma H1355 exposed for 24 hours to equivalent doses of NP or ions (Kao, Y.Y., et al., 2012). Similar observations were also made by Heng et al. When applied doses are below 200 μM of total zinc, BEAS-2B cell mortality induced after 24 h by NPs is identical or close to that obtained with ions at equivalent concentrations. However, above this dose, ions prove more toxic (Heng, B.C., et al., 2011). Nevertheless, other studies, such as that by Xia et al. on the same cell model as well as on RAW 264.7 macrophages, highlight greater toxicity with ions than with NPs, even at low zinc doses (Xia, T., et al., 2008). However, both cell models were exposed for 6 h instead of 24 h. It is therefore possible that this exposure time is insufficient to visualize the cytotoxic effects of NPs, since these potentially require prior internalization by cells, while the Ions can diffuse across cell membranes and thus act much more rapidly. It would therefore be interesting to know the results of equivalent tests performed after 24 hours. Based on our results and data from the literature, we can conclude that, unlike copper nanoparticles, the nanoparticulate form of ZnO (nanopowder only) does not exhibit

significantly greater toxicity than the ionic form of zinc. Therefore, we can expect to observe similar effects between ZnO/H₂O and Zn during proteomic analysis and targeted functional tests. However, we can also assume that the effects of cationic ZnO dispersion will differ, either in the amount of changes observed or in the nature of the proteins and cellular processes affected.

2.1 Proteomic Analysis of the RAW 264.7 Cell Line:

Proteomic analysis of the effects of copper NPs showed that the response of the two macrophage cell lines was broadly similar, with the affected cellular processes and compartments being essentially the same, with only a few differences (a few proteins specific to one or the other cell line). Therefore, given the time constraints, we chose to investigate the effects of ZnO NPs and Zn ions only on the proteome of the RAW 264.7 cell line. As with copper, the hypotheses generated by this analysis were subsequently explored and validated by complementary targeted and functional assays to better elucidate the molecular mechanisms involved in the toxicity of zinc oxide and zinc ions. For this purpose, the cells were exposed as before for 24 hours to nanoparticles or ions with corresponding LD₂₀ values (cationic ZnO at 4 µg/mL; ZnO/H₂O at 8 µg/mL; Zn²⁺ at 120 µM), and then the total proteins were analyzed by 2D electrophoresis. The entire experiment was repeated four times on independent cultures. Image analysis, spot detection, and quantification were performed using Delta2D software. Spots showing statistically significant variations compared to control conditions (same selection criteria as for copper) were analyzed by nanoLC-MS/MS to identify the corresponding proteins against the UniprotKb/SwissProt database. Figure 5 shows the typical gels obtained during this analysis for the different conditions, and an area of this gel has also been enlarged to improve visibility and is shown in Figure 6. The results of the mass spectrometry identification are detailed in Table 2.

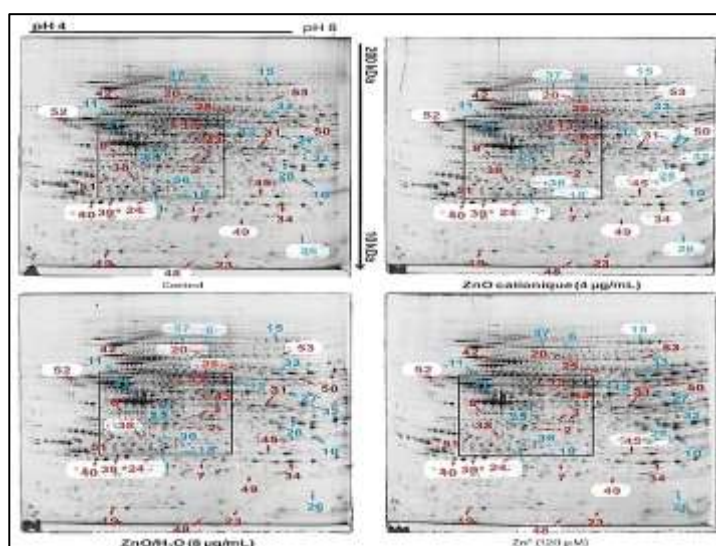


Figure 5: 2D gels obtained for the zinc-treated RAW 264.7 cell line. Cells were exposed to NPs and ions, and then 150 µg of total protein was analyzed by 2D electrophoresis: linear gradient IEF from pH 4-8; 2D (SDS-PAGE) from 10 to 200 kDa; rapid silver nitrate staining.

Quantitative spot analysis was performed using Delta2D software. Red arrows: proteins repressed in at least one condition; blue arrows: proteins induced in at least one condition. Numbers correspond to those in the identification table (Table 2). The central area of the gels has been enlarged for clarity (see Figure 6).

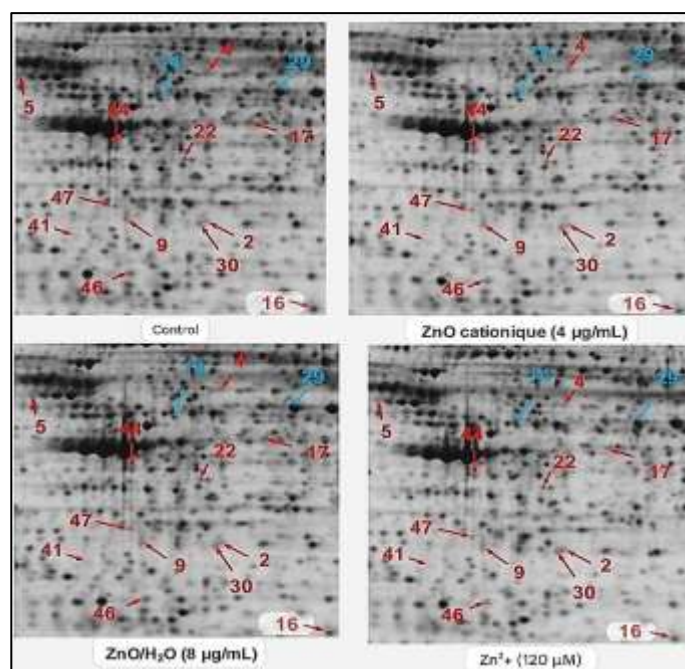


Figure 6: Enlargements of gels obtained for the zinc-treated RAW 264.7 cell line. The area shown corresponds to the boxed central area on the gels in Figure 5. Red arrows: proteins repressed in at least one condition; blue arrows: proteins induced in at least one condition. Numbers correspond to those in the identification table (Table 2).

i. Overall Analysis of Results:

Quantitatively, Delta2D analysis led to the detection of approximately 2200 spots, among which 53 significant variations were identified, representing about 2.5% of the proteome observable on standard gels. As explained in the case of copper, this relatively small proportion strengthens the reliability of the results and suggests that the observed effects are indeed those related to nanoparticles (NPs) or ions, and not solely to cell death processes. This also reinforces the choice of working at the LD20 for conducting the proteomic analysis, since it is sufficient to allow visualization of significant effects without inducing excessive cell death. Furthermore, approximately one-third of the observed changes correspond to proteins whose expression is induced by NP and/or ion treatment, suggesting the activation of certain metabolic pathways in response to the generated stresses. Furthermore, it is also observed that one-third of all modifications concern altered forms of proteins whose major form(s) remain unchanged. This suggests relatively moderate effects of the corresponding variations.

Qualitatively, several major metabolic pathways and cellular compartments were highlighted by this analysis: central metabolism, cell signaling, and protein synthesis and folding, most often corresponding to a general cellular stress response, but also the oxidative stress response, mitochondrial metabolism, the cytoskeleton and intracellular transport, or

even protein degradation, more specific to the response of macrophages to nanoparticles or metal ions (Table 2).

The overall comparison of the effects of ions and NPs at this stage shows that, in the case of zinc, ions have considerable effects: 19 protein forms are affected only by treatment with Zn^{2+} ions (primarily repressions, with only one induction), and 18 are affected jointly with NPs, representing approximately two-thirds of the total changes observed. These proteins are primarily involved in protein synthesis, folding, and degradation, and to a lesser extent in central metabolism, the cytoskeleton, and cell signaling (Table 2). This can be explained by the extensive functions of zinc in the body. Indeed, this essential element often acts as an ion (zinc finger proteins) or as a cofactor for many structural or enzymatic proteins (Prasad, A.S., 2008). It is therefore not surprising that zinc ions have very extensive effects here. Furthermore, these results suggest that ZnO nanoparticles do not necessarily have greater toxicity than ions at equivalent doses, since they do not induce more changes in absolute quantity. However, only detailed analysis of the proteins affected by one or the other treatment will allow us to draw conclusions on this point, as the proteins involved may be different. Of the 16 other protein forms identified, only 4 are commonly affected by both NPs: a mitochondrial aldehyde dehydrogenase (ALDH), a protein involved in alcohol metabolism (repressed, ratio up to 0.678 with $\text{ZnO}/\text{H}_2\text{O}$, $p=0.01$), a glycosyltransferase (DDOST 48kDa) and a translation initiation factor (eIF-4E), involved in protein biosynthesis (induced, ratio up to 1.391 with cationic ZnO, $p = 0.03$, and up to 1.408 with cationic ZnO, $p = 0.04$ with $\text{ZnO}/\text{H}_2\text{O}$, respectively), and an inosine-5'-monophosphate dehydrogenase (IMPDH-2), involved in purine metabolism (repressed, ratio up to 0.676 with Cationic ZnO, $p=0.01$). This latter result also suggests toxic effects of ZnO NPs on nucleic acid synthesis. The other affected proteins, which mainly concern general stress response processes, but also oxidative stress and the cytoskeleton, are distributed equally between the two NPs (5 proteins modified only by cationic ZnO, and 7 by $\text{ZnO}/\text{H}_2\text{O}$), suggesting equivalent toxicity of the two types of particles at their respective LD20s.

These data are broadly consistent with those of the viability tests presented previously: when cells are exposed to doses inducing the same degree of mortality (LD20), the responses observed between ions and nanoparticles on the one hand, and between cationic ZnO and $\text{ZnO}/\text{H}_2\text{O}$ on the other, are of the same magnitude, although the nature and functions of the proteins affected may be different. The cell responses to ionic and nanoparticulate zinc will therefore be presented successively according to the major metabolic pathways affected.

The general response of cells to stress is most often characterized by changes in certain proteins involved in central metabolism, biosynthesis and protein folding, cell signaling, and the cytoskeleton, processes which we have also highlighted in this analysis. Comparing the proteins identified here with those in the list of classically identified proteins, or those already seen in proteomics, reveals some common proteins: α -enolase (ENO-1), triosephosphate isomerase (TPI-1), pyruvate kinase (PKM), phosphoglycerate kinase 1 (PGK-1), and malate dehydrogenase (MDH-1) (central metabolism), but also translation initiation and elongation

factors (eIF-5A-1 and EF-2) and peptidyl-prolyl isomerase A (or cyclophilin A) (protein synthesis), as well as actin and certain forms of Rho-GDI (cytoskeleton) (Petrak, J., et al., 2008; Wang, P., et al., 2009). These proteins represent approximately one-quarter of all the forms identified in these different processes combined. However, the other identified proteins do not appear in the "already seen" list, and similarly, many other proteins from this classic list were not identified here (notably the HSP stress response proteins, or the 14-3-3 family proteins, tubulins, etc.). Therefore, the proteins identified in this analysis can be considered to represent, overall, the response of RAW 264.7 cells to zinc treatment (NP or ions).

In particular, regarding central metabolism, ions and NPs, notably cationic ZnO, induce modified forms of several enzymes in the glycolysis pathway: a form of PGK-1 (ratio up to 1.492, $p = 0.03$ with cationic ZnO), catalyzing the formation of 3-phosphoglycerate from 1,3-bisphosphoglycerate (step 6); a form of PKM (ratio up to 1.466 with cationic ZnO, $p = 0.00$), catalyzing the formation of pyruvate from phosphoenolpyruvate (final step 9) (Dang, C.V., 2013); and a form of α -enolase (ratio up to 1.323 with ZnO/H₂O, $p = 0.04$), an enzyme in the step preceding the one catalyzed by the PKM. With the exception of the latter, these are also mainly minor modified forms for which the major form(s) remain mostly unchanged. It is therefore difficult at this stage to draw definitive conclusions about the effects of these variations, which could be assumed to be moderate. However, although these proteins individually belong to the "already seen", their simultaneous induction by zinc, associated with the induction of all forms of enolase, could nevertheless suggest here an over-activation, even non-specific, of the glycolysis pathway and therefore of the production of pyruvate, itself necessary for the production of ATP at the level of the mitochondria. Furthermore, Zn² ions also induce a decrease in another modified form of PGK-1 (ratio of 0.791, $p=0.04$), simultaneously with an increase in the previously mentioned form, at the limit of statistical significance. In this latter case, since the overall sum of the intensity of these two forms does not vary significantly, and the predominant form is also unaffected by the ions, we can assume that there is only a redistribution of the modified forms of this enzyme, the effect of which on metabolism is difficult to predict at this stage. In parallel, nanoparticles (NPs) and ions also lead to an increase in an aldose reductase (ratio of 1.206 with ZnO/H₂O, $p = 0.01$), an enzyme that enables the formation of fructose from glucose via the polyol pathway, using sorbitol as an intermediate, without energy consumption as occurs in the early stages of glycolysis. This activation could therefore be a way for cells to enhance glycolysis by producing the necessary metabolites through alternative, less energy-intensive pathways.

Furthermore, it appears that this enzyme also plays a role in establishing the inflammatory response in certain cell types (Ramana, K. V., and S. K. Srivastava, 2010). Thus, the induction observed here could also be linked to NP-induced inflammatory mechanisms.

Finally, the slight repression by NPs and ions of a form of cytoplasmic MDH-1 (ratio of 0.820 with ZnO/H₂O, $p=0.01$), involved in the conversion of malate from mitochondria to oxaloacetate destined in this case for gluconeogenesis, the reverse pathway of glycolysis (Kalhan, S.C., et al., 2008; Hanspers, K., et al., 2013), as well as that of a form of TPI-1 by

cationic ZnO (ratio up to 0.832, $p = 0.05$), an enzyme catalyzing the interconversion of glyceraldehyde-3-phosphate to dihydroxyacetone phosphate exiting the glycolysis pathway, could suggest that cells also attempt to limit the consumption of intermediate and final products of glycolysis, in order to ensure its high-rate functioning. Nevertheless, these are still modified forms of the two enzymes, whose major forms.

The major forms are not affected by zinc. Thus, it remains difficult at this stage to draw definitive conclusions about the associated response.

All of these results suggest a reorientation of classical metabolic pathways by several means, with the aim of favoring the glycolytic pathway. This response, which is not truly specific to zinc exposure (essentially "already seen"), would allow cells to compensate for the increased energy requirements, enabling them to combat the stress induced by nanoparticles and, to a lesser extent, ions (often at the limit of statistical significance in this case). However, with the exception of enolase, for which all forms are induced, the absence of changes observed in the major forms of the proteins concerned does not allow for a definitive conclusion. It therefore seems necessary and relevant here to validate or refute this hypothesis by a targeted test involving the enzymatic and colorimetric measurement of the final product of glycolysis: pyruvate. Furthermore, ions, and to a lesser extent ZnO/H₂O, also induce an increase in 6-phosphogluconate dehydrogenase (6-PGDH) (ratio of 1.305 with Zn, $p = 0.04$). This is the third enzyme in the pentose phosphate pathway, an alternative to glycolysis for carbon metabolism, which also allows the production of redox equivalents in the form of NADPH, participating in the regulation of cellular redox balance (Stover, N.A., et al., 2011; Hanspers, K., et al., 2013). It would therefore seem that, in the case of ions, this pathway is preferentially induced over the classical pathway, both as part of a general response to induced stress and to combat potential oxidative stress. This latter hypothesis could be tested by measuring the enzymatic activity of the 6-PGDH.

Finally, regarding the other pathways constituting the general cellular stress response, proteins are predominantly affected by ions on the one hand, and repressed on the other. In the case of cell signaling, this mainly involves G protein subunits (guanine nucleotide-binding proteins) as well as a modified form of a phosphatase, the major form of which remains unchanged. Protein biosynthesis and folding pathways are affected at the level of several transcription, translation initiation, and elongation factors, but also amino acid ligases and tRNAs, a ribosomal protein (No. 21), chaperones (Nos. 16, 23), and other proteins involved in amino acid synthesis (Nos. 22, 24) or protein maturation (Nos. 14, 16). This reflects either a toxicity of Zn ions, and to a lesser extent of ZnO NPs, that the cells are unable to compensate for, or the fact that, as before, the cells concentrate their efforts on the processes necessary to combat stress, thus inducing a slight decrease in the activity of other cellular processes.

iii. Analysis of the oxidative stress response:

The response of RAW 264.7 cells to oxidative stress is specific to the treatments applied to the cells, since the observed modifications are not among the proteins classically identified in proteomics ("already seen") (Petrak, J., et al., 2008; Wang, P., et al., 2009). However, this

response is very limited here, even though we studied metallic nanoparticles and zinc ions, known to interact with a large number of elements within the cell. Indeed, it is primarily characterized by a very strong induction of the regulatory subunit of glutamate-cysteine ligase (GCLM), both with nanoparticles and ions (ratio up to 1.837 with Zn, $p = 0.03$). Since this enzyme is involved in the first step of glutathione synthesis (the rate-limiting step), this result suggests increased glutathione production in cells to compensate for the induced stress. To validate this hypothesis, the intracellular glutathione level was measured using a targeted assay. While the effects of ZnO NPs on glutathione or the enzymes involved in its synthesis are poorly described, those on ions, on the other hand, are better documented. It has already been shown, in various cell models, that a zinc deficiency can reduce the amounts of GCLM and glutathione (Omata, Y., et al., 2013), which could suggest that, conversely, an increase in zinc can indeed induce an increase in the synthetic enzyme and the final product, a phenomenon also observed by some groups (Ha, K.N., et al., 2006). However, other studies suggest that this increase is only transient, and that the amounts of glutathione and GCLM subsequently decrease over time, as does the activity of glutathione reductase, a key enzyme in GSH regeneration (Wilhelm, B., et al., 2001). These results can be interpreted as follows: (1) a zinc deficiency reduces glutathione production, making cells more susceptible to potential oxidative stress; (2) at normal physiological doses, zinc stimulates glutathione synthesis and thus appears to have a protective and, indirectly, antioxidant effect, necessary for the cell to maintain its redox balance and combat potential stress; (3) conversely, if zinc is present in excess, it initially retains its antioxidant role, over-activating glutathione synthesis. However, if the zinc dose becomes excessive, it becomes truly toxic and eventually induces cell death, associated with a decrease in GSH production.

Furthermore, two other changes were observed within the context of this response. First, the heme oxygenase isoform 2 (HO-2) was significantly repressed only by ZnO/H₂O nanoparticles (ratio of 0.492, $p = 0.02$). Heme oxygenases catalyze the degradation of hemes in microsomes, releasing iron, carbon monoxide (CO), and biliverdin, which can then be converted to bilirubin. They are generally associated with the oxidative stress response, which can be explained by the fact that, on the one hand, bilirubin and biliverdin have potential antioxidant properties, and on the other hand, that CO could act similarly to NO, capable of activating signaling cascades involved in this type of response (Kikuchi, G., et al., 2005). The observation of this modification therefore appears consistent with the literature. In contrast, the most frequent response, which was not observed here, is the induction of HO-1, particularly by heavy metals, while HO-2 is constitutively expressed and therefore can only be repressed if affected. In this context, one could therefore assume that ZnO has toxic effects forcing the cell either to repress HO-2, through direct interaction with zinc or to limit the release of metals from hemes, which could further disrupt the redox balance, or to inhibit this potential antioxidant pathway to favor the main pathway involving glutathione. However, an earlier study seems to corroborate the fact that zinc is toxic and can decrease the HO-inducing capacity (Drummond, G.S., and A. Kappas, 1979). Finally, the ions repress neutrophil cytosol factor 4 (NCF-4) (ratio of 0.530, $p = 0.03$), a regulatory protein contributing to the assembly

of the NADPH oxidase complex, responsible for the oxidative surge through the formation of ROS (particularly superoxide ions) (Lee, R., et al., 2012). The repression of this factor could be linked to the coupling between the toxic and antioxidant effects of zinc ions, and would aim to limit the oxidative burst in order to protect cells against excessive stress. This mechanism would then complement the glutathione overproduction highlighted above.

iv. Analysis of the mitochondrial response:

In general, the effects of zinc on mitochondria remain limited, characterized by only three modifications. Furthermore, two of these modifications are frequently observed in proteomic analysis: aldehyde dehydrogenase (ALDH) and the β subunit of an ATP synthase (ATP-5B) (frequencies of approximately 15% and 20%, respectively) (Petrak, J., et al., 2008; Wang, P., et al., 2009). The observed response is therefore not specifically linked to ZnO nanoparticles and zinc ions. Nevertheless, understanding the mechanisms involved remains necessary and of interest.

First, we observe the repression of ALDH by both NPs (ratio up to 0.678 with ZnO/H₂O, $p = 0.01$). This mitochondrial matrix protein is involved in alcohol metabolism: acetaldehyde, produced from ethanol in the cytosol, is taken up by ALDH in the mitochondria and converted to acetate, which is then used to form acetyl-CoA, which enters the Krebs cycle. However, acetate formation is accompanied by the production of reduced equivalents in the form of NADH, which, at the mitochondrial level, can lead to the production of ROS (Brocardo, P.S., et al., 2011). Here, the repression of ALDH could therefore be interpreted as a further attempt by cells to reduce ROS production and combat oxidative stress. Furthermore, two proteins of the inner mitochondrial membrane are also modified, both by ions and NPs: a modified form of ATP-5 α (repressed, ratio up to 0.355 with Zn, $p = 0.02$), involved in energy production in the form of ATP in the presence of a transmembrane proton gradient, and a modified form of the mitochondrial inner membrane protein, also known as name of mitofilin (induced, ratio up to 1.645 with ZnO/H₂O, $p=0.01$), involved in the structural maintenance of mitochondrial membranes, particularly the structure of the inner membrane cristae (von der Malsburg, K., et al., 2011; Zerbes, R.M., et al., 2012). Although these modifications are very intense, they are once again variations affecting modified forms, with the major form of the protein remaining unchanged in both cases. This suggests that the effects of the observed variations will likely be very moderate. However, if effects are visible, they could be considered structural changes in mitochondria under the influence of zinc (NP and ions), associated with possible variations in the transmembrane potential, which is strongly linked to the proton gradient. We therefore investigated this hypothesis nonetheless, by measuring variations in transmembrane potential using a targeted technique based on flow cytometry. Furthermore, since the repression of ATP synthase subunits can, by extension, lead to a decrease in ATP production, these results would also be consistent with the possible overactivation of central metabolism, which would then reflect the effort made by cells to compensate for energy losses and combat stress.

v. Effects of zinc on the cytoskeleton and intracellular transport:

The cytoskeleton is also affected by zinc (NPs and ions), both structurally and functionally. Furthermore, among the 8 observed modifications, 2 are "already seen in proteomics": a form of actin (slightly increased with ZnO/H₂O, ratio of 1.270, $p = 0.03$), and a modified form of Rho-GDI-1, controlling the disassembly of actin filaments (slightly repressed with Zn, ratio of 0.866, $p = 0.01$), for which the major forms are unchanged (Petrak, J., et al., 2008; Wang, P., et al., 2009). These modifications could nevertheless suggest moderate structural changes in cells, non-specific to zinc exposure, while the other modified proteins correspond to the specific response of cells to ZnO or Zn² ion.

In particular, we note the induction by NPs of gelsolin, also called "actin-depolymerizing factor" (ADF) (ratio of 2.307 with ZnO/H₂O, $p = 0.04$) and of a subunit of the "F-actin-capping protein" (ratio of 1.427 with cationic ZnO, $p = 0.02$). These two proteins are involved in the regulation of actin filament polymerization, which is Ca-dependent in the case of gelsolin.

Moreover, for In the latter, all spots corresponding to the modified forms of the protein are induced in the same way, at the limit of statistical significance (data verified but not shown). This again suggests the induction of structural changes in cells by NPs.

Furthermore, repression of a modified form of Rho-GDI-2 is observed, both by NPs and ions (ratio up to 0.752 with ZnO/H₂O, $p=0.04$), which can be associated with the repression of the Rho-GDI-1 form mentioned above. However, since the major forms of these two Rho-GDI proteins are unchanged, it remains difficult to draw definitive conclusions about the effects of these modifications. Nevertheless, when combined with those of structural or regulatory proteins involved in the assembly and disassembly of actin filaments, they could still suggest a potential impact on phagocytic processes, which involve these filaments. Indeed, Rho-GDI inhibition would prevent the regulation of actin filament depolymerization, potentially leading to the formation of stress fibers and a reduction in the phagocytic capacity of cells (O'Reilly, P.J., et al., 2003). We therefore chose to evaluate these potential effects using a targeted functional assay to validate or refute this hypothesis.

Furthermore, our results suggest that ions also induce structural changes, but through different mechanisms. Indeed, while proteins constituting or regulating actin filaments are not significantly affected by Zn², a slight repression of a protein from the RP/EB family, which regulates microtubule assembly, is observed (ratio of 0.809, $p = 0.03$). The toxic effects of ionic zinc, as well as its interaction with tubulin but not with actin, have been documented for several years (Kress, Y., et al., 1981; Rudolf, E., et al., 2008; Mackenzie, G.G., and P.I. Oteiza, 2007).

These results therefore appear consistent with the literature. Interestingly, we also observed here modifications (repressions) of certain proteins involved in intracellular trafficking, particularly under the influence of ions. First, the SNAP- α protein (α -soluble NSF attachment protein), required for vesicular transport between the endoplasmic reticulum and the Golgi apparatus, is repressed by the Zn² ion only (ratio of 0.711, $p = 0.03$). This result suggests a potential inhibition of intracellular protein transport pathways. Furthermore,

cationic ZnO NPs and ions also affect the SNX-2 protein ("sorting nexin-2") (repression of a modified form with a ratio of up to 0.839 with Zn, $p = 0.02$, and similar but non-significant repression of other forms). SNX proteins play a role in endocytosis mechanisms by participating in the control of the destination of compounds contained in early endosomes (Carlton, J., et al., 2004). Indeed, these compounds can (1) be retained in these vesicles until their maturation into late endosomes and then into multivesicular bodies, followed by their fusion with lysosomes (degradation pathway); (2) be redirected to the cell membrane after their retrieval by the "endocytic recycling compartment" (recycling pathway); (3) be transiently directed to the recycling compartment and then taken up by a transport complex regulated by SNX proteins (Gullapalli, A., et al., 2004; Carlton, J.G., et al., 2005), which then transports them to the Golgi apparatus (non-degradation pathway).

Macrophages can employ classical endocytosis mechanisms, but also macropinocytosis, a non-specific, actin-dependent form of endocytosis that allows the internalization of compounds ranging in size from a few hundred nanometers to 1 micrometer in solution in an extracellular fluid (Lim, J.P., and P.A. Gleeson, 2011). Like phagocytosis, it plays a key role in their function of eliminating pathogens or foreign bodies. The choice of one pathway or the other depends primarily on the size of the element to be internalized. These results, combined with those possibly involving phagocytosis, suggest even moderate disruptions of several internalization pathways under the influence of zinc, both nanoparticulate and ionic. More specifically, in the case of macropinocytosis, which can be considered for the NPs studied here given their size (approximately 200 to 250 nm), the inhibition of the control of the non-degradation pathway could be interpreted as a mechanism implemented by cells to preferentially direct NPs towards lysosomal degradation. Such mechanisms for redirecting internalization pathways have already been demonstrated in the case of non-metallic NPs (Kadiu, I., et al., 2011). Moreover, this result would be consistent with the electron microscopy analyses presented earlier (Figure 2), in which it can be seen that some of the internalized NPs are very small, than the aggregates of the suspension initially used to treat the cells, suggesting intracellular degradation.

vi.. proteins affected by zinc:

Some proteins involved in purine metabolism were moderately affected. repressed with both NPs (ratio up to 0.676 with cationic ZnO, $p 0.01$). These two proteins are involved in purine metabolism (AMP and guanine synthesis, respectively). This result suggests that ZnO NPs could have detrimental effects on nucleic acids, which the cells would no longer be able to repair. These effects could be assessed by targeted tests such as the comet assay.

3. Results

As in the study of the effects of copper, several types of targeted tests were performed to establish correlations between the observed molecular variations and functional events, in order to further investigate and validate the hypotheses established from the proteomic analysis data. These tests were primarily based on flow cytometry techniques, but also included biochemistry and enzymology.

3.1. Effects of Zinc on the Phagocytic Capacity of Cells:

Proteomic analysis shows that zinc, particularly in its ionic form, affects some cytoskeletal proteins, often indirectly involved in phagocytosis mechanisms. As with copper, this test was therefore evaluated after treatment of the cells with zinc and then contact with 1 μm fluorescent beads. The objective here is to determine whether macrophages are still able to phagocytose exogenous compounds after zinc exposure. The effects of cationic ZnO and ZnO/H₂O nanoparticles, as well as Zn²⁺ ions, were evaluated on the two macrophage cell lines at their respective LD20 values. This test was performed under the experimental conditions developed during the copper study (notably the D-sorbitol desorption step). The phagocytosis rate (fluorescence of internalized beads) was then evaluated by flow cytometry (FACScalibur) using the FITC excitation/emission parameters (495 nm/520 nm). The results obtained are presented in Figure 7. First, it should be noted that the background noise (adsorption rate of beads on the cell surface at 4°C) is relatively low ($\leq 5\%$ in all tests), with the main peak observed between 10° and 10' intensity corresponding, as before, to cell autofluorescence. Thus, the fluorescence measured under the tested conditions is indeed solely that of internalized beads. Furthermore, these tests showed that, in the absence of zinc treatment, 70 to 90% of the cells are phagocytic, most being capable of internalizing at least one and up to four or more beads, resulting in a corresponding number of intensity peaks. This internalization of beads is also accompanied by a slight increase in cell granularity (see 37°C control, Figure 7, left). The high proportion of phagocytic cells observed highlights the importance of this function in macrophages.

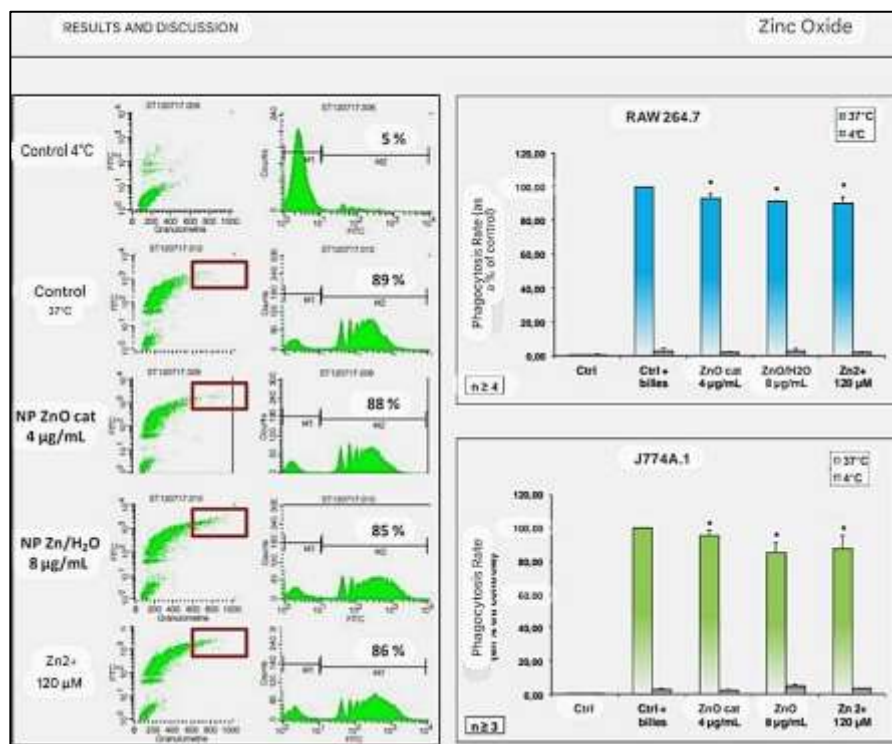


Figure 7: Effects of zinc on the phagocytic capacity of RAW 264.7 and J774A.1 cell lines.

Cells were treated for 24 h with nanoparticles or ions at their LD20, then incubated with fluorescent beads for 2 h at 37°C or 4°C. The fluorescence of internalized beads (live cells only)

was measured using FACScalibur (FITC filters). Left: Dot plots and typical histograms obtained for the different conditions. Right: Mean phagocytosis rate under the different conditions, expressed as a percentage of the control at 37°C. Data were analyzed using the Mann-Whitney U nonparametric test: $p \leq 0.05$

In the case of cells treated with NPs at their respective LD20, a slight increase in cell granularity is observed, more pronounced with ZnO/H₂O than with cationic ZnO, suggesting that the particles have been internalized by the cells (Figure 7, left, boxed in red). However, this internalization does not induce a marked decrease in phagocytosis (Figure 7, right). Indeed, although the slight decrease observed is statistically significant ($p \leq 0.05$), it corresponds to only a 5–10% loss of total phagocytic capacity. This suggests that cells that have ingested ZnO NPs are still capable of internalizing other elements. This appears to be consistent with the proteomic analysis data, which showed slight variations in some modified forms, often minor, of proteins involved more or less directly in phagocytosis mechanisms, thus suggesting possible, but very moderate, disruptions to these mechanisms. Furthermore, when cells are exposed to Zn² ions at the corresponding LD20 (120 µM), a slight increase in granularity is also observed (Figure 7, left, red box), without significant effects on phagocytosis, as in the case of NPs. These observations are also consistent with those of the proteomic analysis, in which the ions often had effects similar to those of NPs. Moreover, since the increase in granularity is linked to an increase in overall cellular complexity, it could be assumed that this increase is related to the disruptions of intracellular trafficking mechanisms suggested by the proteomic analysis (endocytosis, macropinocytosis), mechanisms which also involve the movement of intracellular vesicles. Furthermore, we observe here that both macrophage lines respond in the same way to both ions and nanoparticles, which reinforces the validity of the observed effects. All of these data therefore allow us to conclude that when macrophage lines are exposed to zinc, they undergo a limited number of molecular alterations at the cytoskeletal level, mainly affecting non-majority modified protein forms, which are insufficient to disrupt the cells' ability to phagocytose other elements. Moreover, since the size of the beads is close to that of bacteria, we can also consider that zinc, whether in nanoparticle or ionic form, does not impair innate immune defenses.

3.2. Effects of zinc on glutathione:

Proteomic analysis showed that zinc (NPs and ions) induces a relatively moderate oxidative response: classic oxidative stress response proteins are not affected (peroxyredoxins, heme oxygenase 1, etc.), and only a strong induction of GCLM is observed, both with NPs and ions, suggesting an overactivation of glutathione production in an attempt to return to a normal redox state. It therefore seemed relevant here to measure intracellular glutathione levels, as was done for copper, using the same method based on the use of monochlorobimane (MCB). The cells were exposed to NPs and zinc ions, then labeled with MCB, which specifically detects reduced glutathione (GSH). The fluorescence of the complex formed was measured by flow cytometry (MoFlo), and the results are shown in Figure 8.

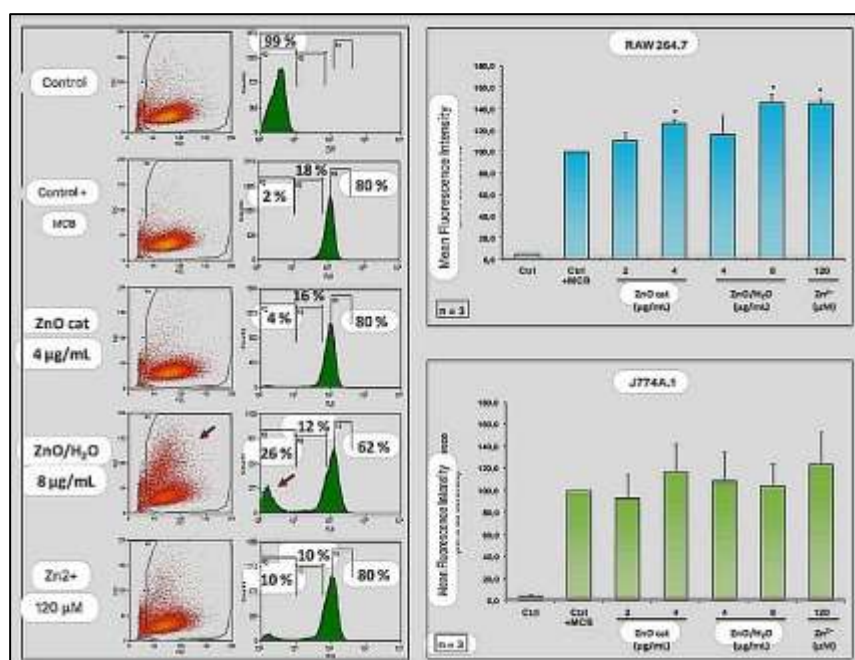


Figure 8: Effects of zinc on intracellular reduced glutathione in RAW 264.7 and J774A.1 cell lines.

Cells were treated for 24 h with NPs or ions at different doses, then labeled with MCB for 5 minutes at 37°C. Live cell fluorescence was measured with MoFlo (Excitation/Emission: 350/450 nm). Left: Typical dot plots and histograms obtained for the different conditions. Right: Mean fluorescence intensity under the different conditions, expressed as a percentage of the control. Data were analyzed using the Mann-Whitney U nonparametric test: * $p \leq 0.05$

First, in the absence of zinc and MCB treatment, cells are mostly detected in a low-intensity main peak (between 10^0 and 10^1), corresponding to cell autofluorescence. MCB treatment induces a shift in this main peak to an intensity of 10, corresponding to the majority of cells (80% in the example shown, Figure 8, left), but also to the maximum total maximum total of GSH present in cells under normal conditions. Minority populations containing lower levels of glutathione are also detected. When cells are exposed to cationic ZnO at doses less than or equal to the LD20 (4 $\mu\text{g/mL}$) and then labeled with MCB, the same profiles are obtained as in the case of untreated cells, with a very slight increase in the cell population with lower glutathione levels (Figure 8, left). This suggests that any effects of this type of NP are rapidly controlled by the cells. Thus, the induction observed in proteomic analysis with these NPs for GCLM, the regulatory subunit of the glutathione-synthesizing enzyme, could here allow an increase in GSH production in sufficient quantities, or more, to compensate for any losses related to the NPs. Indeed, the analysis of the two cell lines (Figure 8, right) shows no significant decrease in the overall GSH level, and in the case of RAW 264.7, this level is even slightly increased, which could be related to the induction of GCLM. This compensation suggests a low-intensity stress, to which the J774A.1 cell line appears to be slightly more sensitive than the RAW 264.7 cell line, as previously observed. Exposure to ZnO/H₂O at the LD20 (8 $\mu\text{g/mL}$) induces a 25% decrease in the main population with high GSH levels (Figure 8, left), associated with a slight increase in size and granularity, visible in the dot-plot

representation (red arrows). This reflects the fact that the NPs were internalized by the cells, which is consistent with electron microscopy analyses and targeted phagocytosis assays. Once internalized, the NPs are then able to decrease glutathione levels in approximately one-quarter of the cell population. However, this also means that the majority of cells are able to resist the induced stress, which analyses on both cell lines confirm (Figure 8, right): GSH levels are stable in cell line J774A.1 and increase slightly in cell line RAW 264.7, probably due to the increase in GCLM associated with NP-induced oxidative stress. Thus, as with cationic ZnO, and although the analysis profiles show more intense effects, the oxidative stress induced by ZnO/H₂O is also rapidly managed by the cells.

Finally, when exposed to Zn ions, the population corresponding to the lowest GSH level is slightly increased (10% instead of 2% in untreated cells), and the populations of intermediate levels are slightly decreased (10% instead of 18%). However, the main population of the highest GSH level remains unchanged (Figure 8, left). Thus, it appears that there is only a redistribution of the low GSH population, with the majority of cells retaining a high amount of glutathione, suggesting moderate oxidative effects from the ions. Indeed, analysis of both cell lines shows that the cells also do not undergo marked changes in their glutathione level, which is even increased in both lines, more significantly in RAW 264.7 again. Once again, this increase is assumed to be related to the induction of GCLM. All the results obtained here are therefore consistent with those of the proteomic analysis. In particular, the increase in GCLM detected in all three conditions appears to induce GSH overproduction in response to oxidative stress, sufficiently to maintain stable glutathione levels in J774A.1 cells, and to induce an increase in these levels in RAW 264.7 cells, which is consistent with the increased sensitivity of the J774A.1 cell line. Furthermore, the induction observed in proteomic analysis was stronger with ions than that observed with ZnO/H₂O, which was itself slightly stronger than that observed with cationic ZnO. The analyses performed here are in perfect agreement with these observations, with the increase in glutathione levels also following the same trends ($\text{Zn}^{2+} \geq \text{ZnO}/\text{H}_2\text{O} \geq \text{cationic ZnO}$). In all cases, the oxidative stress induced by zinc (NP and ions) thus appears to be moderate, and this increase in GSH seems to allow cells to respond sufficiently, which also corroborates the small amount of changes observed at the molecular level for classical proteins responding to this type of stress. Once again, it seems worthwhile to confirm these results in primary cells in order to draw definitive conclusions on this parameter.

3.3. Effects of Zinc on NO Production:

To complement the study of the effects of zinc, we also evaluated the effects of ZnO NPs and Zn ions on the ability of macrophages to produce inflammatory mediators. Indeed, proteomic analysis did not show, a priori, any effects of zinc on proteins involved in the inflammatory response of macrophages, and one might therefore expect that zinc would not affect these processes. Nevertheless, analyzing this capacity remains essential, as it constitutes a key function of this cell type, necessary and indispensable for innate immune defenses. The ability of macrophage cell lines to produce NO, after LPS stimulation, was

therefore evaluated here in the presence or absence of zinc (NP and ions), using the Griess method, under the same experimental conditions as in the copper study. The results of this test are shown in Figure 9.

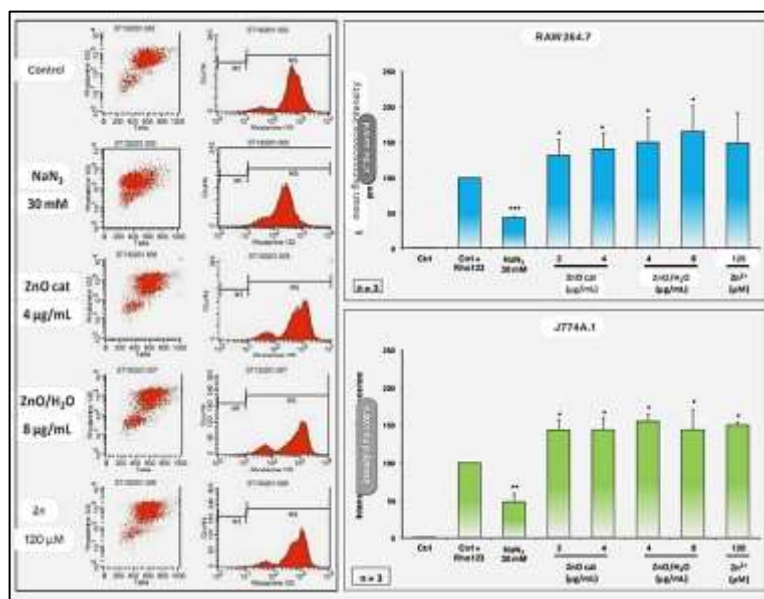


Figure 9: Effects of zinc on NO production by RAW 264.7 and J774A.1 cell lines.

Cells were treated or untreated for 24 h at 37°C with NPs or ions at their LD20, then stimulated or unstimulated for 24 h at 37°C with LPS (1 µg/mL) in the presence of arginine (5 mM). NO secreted into the culture medium was measured by incubating the supernatant with an equivalent volume of Griess's reagent for 30 minutes at room temperature. The absorbance of the complex formed was measured spectrophotometrically at 540 nm against a range of sodium nitrite (NaNO_2). The rate of NO production under the different conditions is expressed as a percentage of the stimulated control. Statistical significance according to the nonparametric test of Mann and Whitney: ** $p \leq 0.01$; *** $p \leq 0.001$.

First, this test shows that cells only produce NO after stimulation with LPS, simulating the conditions of a pathogen attack. The question then arises as to whether or not zinc affects the cells' ability to respond to such conditions. However, we observe that both types of nanoparticles induce only a slight decrease in NO production capacity: although this decrease is statistically significant in the RAW 264.7 cell line, it remains only around 15%, and no significant decrease is observed in J774A.1. We can therefore deduce that zinc in nanoparticle form does not alter the ability of macrophages to produce this type of inflammatory mediator. This nevertheless corroborates the proteomic analyses, in which no changes were observed for proteins that would be involved in the production of mediators or in establishing the inflammatory response in general. In contrast, ions have very marked effects on NO production: at a dose equivalent to that of NPs ($\text{ZnO}/\text{H}_2\text{O}$), a significant 90% loss in the capacity to produce this mediator is observed in both cell lines. However, proteomic analysis showed no ionic zinc-induced changes in proteins involved in activating the inflammatory response or producing NO. Nevertheless, these results appear consistent with data from the literature. Indeed, the review by Pluth et al. confirms the existence of close interactions

between Zn and NO, particularly in the immune system (Pluth, M.D., et al., 2011). More specifically, numerous studies on various cell models (lung cells, keratinocytes, etc.) demonstrate that zinc, at concentrations of approximately 10 to 150 μM , possesses anti-inflammatory properties and is notably capable of inhibiting the activity of NO synthesis enzymes. This role is also evident in the fact that NO itself, under inflammatory conditions, is thought to induce the release of zinc from metallothioneins, thereby exerting negative feedback on the inflammatory response. Consequently, it has also been shown that zinc deficiency induces significant dysfunctions of the immune system, potentially leading to states of chronic inflammation. Thus, ionic zinc is necessary for the proper conduct and resolution of the inflammatory response, but at excessive concentrations, as appears to be the case in the test performed here, it inhibits the production of NO necessary for this response. This effect is specific to the ionic form of zinc, and it appears necessary to validate these observations by performing the same test on primary cells.

3.4. Effects of zinc on metabolism: pyruvate assay:

Proteomic analysis of the cell lines showed the induction, under the effect of zinc, of several enzymes in the glycolysis pathway, and in particular of all forms of α -enolase, the enzyme of the penultimate step of this pathway, leading to pyruvate production. These observations suggest an activation of central metabolism, within the framework of a zinc-specific response, since these modifications do not correspond solely to those classically observed ("already seen in proteomics"). To validate this hypothesis, pyruvate production by cells in the presence of zinc was evaluated. For this purpose, cells of the RAW 264.7 cell line were exposed to nanoparticles and zinc ions, then lysed. Pyruvate was measured in the lysis supernatants using an enzyme kit, after removal of proteins that, according to the supplier, could interfere with this measurement.

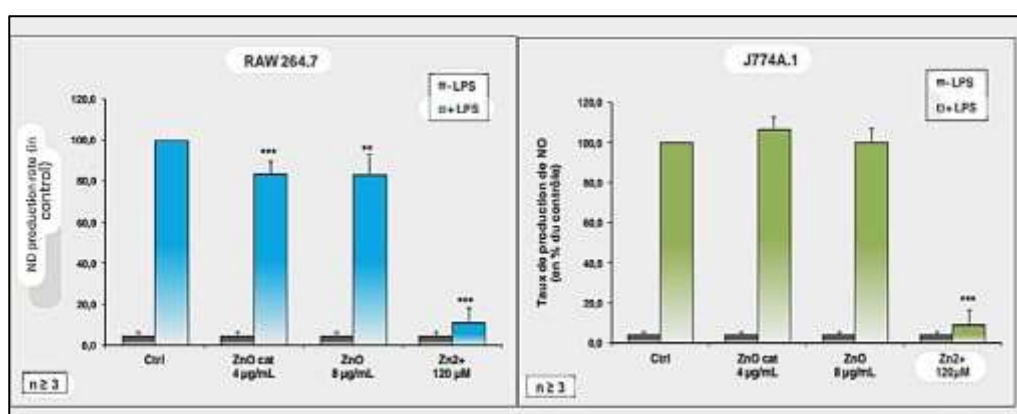


Figure 10: Effect of zinc on pyruvate production by the RAW 264.7 cell line.

Cells were exposed for 24 h at 37°C to NPs and ions at their respective LD20s, then harvested and lysed. The amount of pyruvate produced was then determined enzymatically under the different conditions, leading to the formation of a colored compound proportional to the pyruvate present in the sample, the absorbance of which was measured at 560 nm. The amount of pyruvate was expressed as a function of the number of cells in the sample. Statistical significance according to the non-parametric Mann-Whitney U test: * $p \leq 0.05$.

The results of this test (Figure 10) show that zinc, whether in ionic or nanoparticulate form, does indeed induce a very marked increase in pyruvate production by macrophages: this can be up to three times greater than that obtained under normal conditions ($\text{ZnO}/\text{H}_2\text{O}$ and Zn^{2+}). Thus, our results corroborate those of the proteomic analysis, and we can deduce here that zinc induces an overactivation of the central metabolism of cells, which is assumed to be necessary to combat induced stress. Confirmation of these observations on primary cells also seems interesting here.

4. Discussion

As explained in the copper study, primary cells are the closest model to reality in physiological terms. Although the small amount of biological material obtainable from primary cultures does not allow their use for proteomic approaches such as that performed in this work, primary cells can be used for smaller-scale testing. Here, primary macrophage cultures established from mouse bone marrow were differentiated, characterized, and exposed to nanoparticles and zinc ions for several types of targeted assays, with the aim of confirming the data obtained from cell lines and validating those from proteomic analysis.

4.1. Zinc Cytotoxicity on Primary Cells:

To study the effects of ZnO NPs and Zn ions on primary macrophages, it was first necessary to assess their toxicity to these cells and determine the LD₂₀ to be used in the various targeted assays to follow. For this purpose, the cell death rate in the presence of NPs and ions was estimated by the dye exclusion test, as previously done on cell lines.

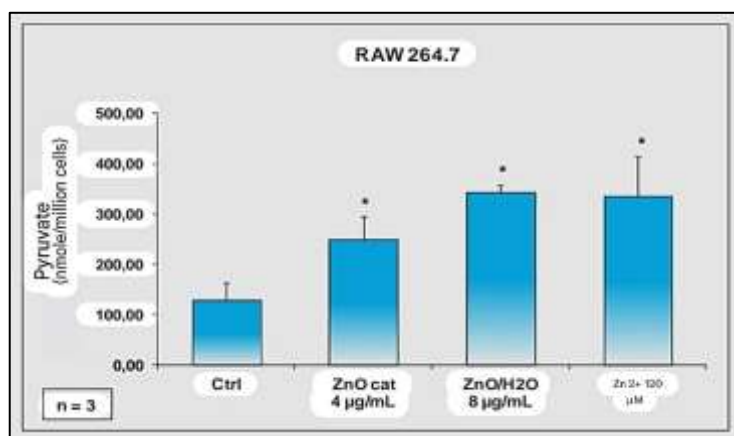


Figure 11: Cytotoxicity of zinc (NPs and ions) on primary mouse macrophages.

Cells were exposed for 24 h to different doses of cationic ZnO , $\text{ZnO}/\text{H}_2\text{O}$, and Zn ions. The death rate was then determined by the Trypan Blue exclusion test. Statistical significance: for $p \leq 0.05$, according to the non-parametric Mann-Whitney U test.

The results (Figure 11) show that the primary cells have the same sensitivity as the cell lines to $\text{ZnO}/\text{H}_2\text{O}$ (LD₂₀ identical to 8 µg/mL), but are less sensitive to cationic ZnO , for which the LD₂₀ also appears to be around the dose of 8 µg/mL, twice as high as that obtained in the two cell lines. These cells are also slightly more sensitive to Zn^{2+} ions: the mortality rate induced at the dose of 120 µM, equivalent to the amount of ionic zinc that could be released

by complete dissolution of the NPs, corresponds well to an LD20 here, whereas this was not quite reached in the cell lines. Thus, at equivalent doses, nanoparticles (NPs) and zinc ions have the same degree of cytotoxicity on primary cells, suggesting equivalent effects of ions and NPs, as has been widely observed in proteomic and cell line-targeted analyses. Differences in sensitivity can again be explained by physiological differences between primary cells and cell lines, particularly the tumor origin of the latter.

4.2. Effects of zinc on phagocytic capacity:

As with the cell lines, the phagocytic capacity of the cells in the presence of zinc was analyzed by flow cytometry. For this purpose, primary macrophages were exposed to nanoparticles and ions, then placed in contact with 1 μm beads, whose fluorescence was subsequently measured. These tests were performed under the same experimental conditions as for the cell lines (with desorption using D-sorbitol to limit background noise). The results of these tests are presented in Figure 12. As with the RAW 264.7 and J774A.1 cell lines, D-sorbitol desorption allowed us to work with limited background noise, although it was slightly higher than with the cell lines (5 to 8% adsorption, data not shown here). As explained in the case of copper, this could be related to the strong adhesion capabilities of the primary cells, which would promote interactions between the cells and the beads pre-incubated in serum. Nevertheless, this value can be considered reasonable, especially since the phagocytosis rate of untreated cells is also higher (generally greater than 80%).

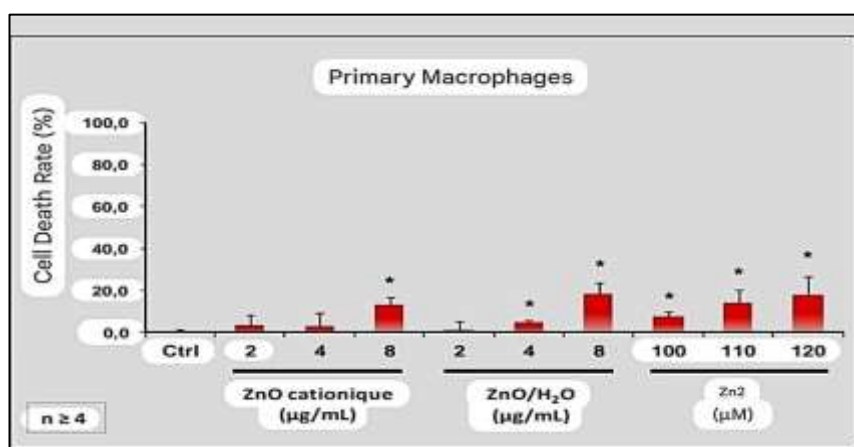


Figure 12: Effects of zinc on the phagocytic capacity of primary macrophages.

Cells were treated for 24 h with nanoparticles or ions at their LD20, then incubated with fluorescent beads for 2 h at 37°C or 4°C. The fluorescence of internalized beads (live cells) was measured using FACScalibur (FITC filters). The mean phagocytosis rate under the different conditions is expressed as a percentage of the control at 37°C. Statistical significance according to the non-parametric Mann-Whitney U test: $p \leq 0.05$. As observed in cell lines, exposure of cells to both nanoparticles and ions, at equivalent doses, does not induce a marked decrease in phagocytosis, although it is statistically significant ($p \leq 0.05$). The loss of phagocytic capacity is only on the order of 5 to 10%, depending on the case (Figure 12). All of these results therefore confirm those obtained in cell lines and thus validate the observations of the proteomic analysis: we can now conclude that zinc nanoparticles and Zn^{2+} ions induce

slight modifications at the level of cytoskeletal proteins (modified forms, not predominant, and mainly involved indirectly in phagocytosis mechanisms). However, the magnitude of these modifications at the molecular level proves insufficient to affect the phagocytic capacity of macrophages and, by extension, to alter innate immune defenses

4.3. Effects of Zinc on Glutathione:

As with the cell lines, the levels of reduced intracellular glutathione in cells in the presence of zinc were analyzed by flow cytometry. For this purpose, primary macrophages were exposed to NPs and ions, then labeled with monochlorobimane (MCB), the fluorescence of which was subsequently measured. These tests were performed under the same experimental conditions as for the cell lines, and the results obtained are shown in Figure 13.

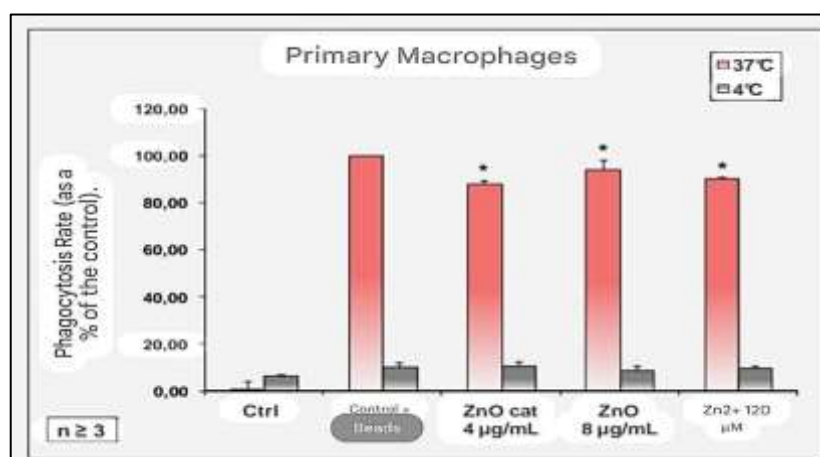


Figure 13: Effects of zinc on reduced intracellular glutathione in primary macrophages.

Cells were treated for 24 h with either NP or ions at different doses, then labeled with MCB for 5 minutes at 37°C. Live cell fluorescence was measured with MoFlo (Excitation/Emission: 350/450 nm). The mean fluorescence intensity under the different conditions is expressed as a percentage of the control. Statistical significance according to the non-parametric Mann-Whitney U test: * $p < 0.05$. As observed in the cell lines, exposure of the cells to both NPs and ions, at equivalent doses, does not induce a marked decrease in intracellular GSH levels. Indeed, although it is statistically significant overall ($p \leq 0.05$) and slightly more pronounced than in the cell lines, this decrease is of the same magnitude under the different conditions and remains in the range of 10 to 20% depending on the case (Figure 13). All of these results therefore confirm those obtained in the cell lines and thus validate the observations of the proteomic analysis: we can now conclude that zinc NPs and Zn^{2+} ions induce slight modifications in oxidative stress response proteins (primarily GCLM induction to increase de novo glutathione synthesis), reflecting low-intensity oxidative stress. The magnitude of these molecular-level modifications is indeed sufficient to rapidly compensate for and control the induced stress and maintain relatively stable GSH levels, and therefore does not justify the establishment of other oxidative response mechanisms involving additional classical proteins of this type.

4.4. Effects of zinc on mitochondrial transmembrane potential:

As with cell lines, potential variations in mitochondrial transmembrane potential were assessed in primary macrophages using the assay based on the internalization of rhodamine 123 by mitochondria.

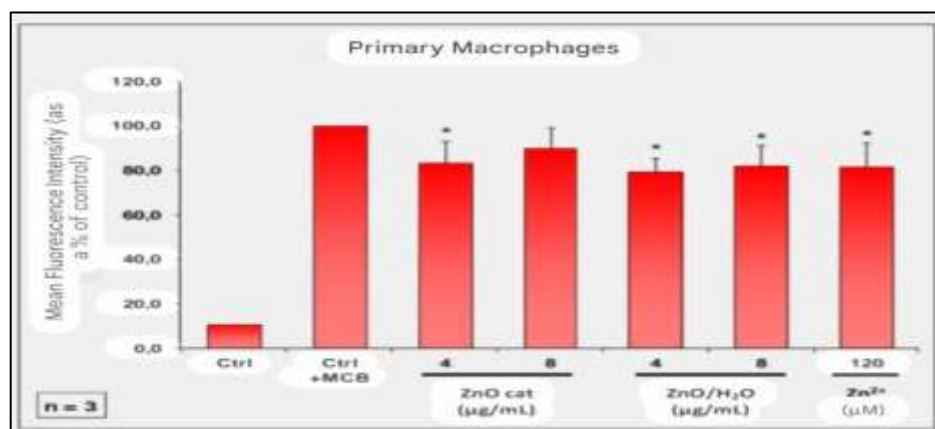


Figure 14 presents a summary of the results obtained

Figure 14: Effects of zinc on the mitochondrial transmembrane potential of primary macrophages. Cells were treated for 24 h with cationic ZnO, ZnO/H₂O, or ions at different doses, then labeled with rhodamine 123 for 30 min at 37°C. Live cell fluorescence was measured with FACScalibur (FITC filters). The mean fluorescence intensity under the different conditions is expressed as a percentage of the control. Statistical significance according to the nonparametric Mann's test. et Whitney: ps0,05; ** p<0,01.

First, it is observed that, unlike the cell lines, the fluorescence intensity, and therefore the mitochondrial transmembrane potential, does not tend to increase in the presence of zinc, whether in the form of ions or nanoparticles: the potential is either stable or slightly decreased (Figure 14). Cationic ZnO induces a slight and non-significant average decrease in this potential at the LD20 (8 µg/mL). The decrease is also observed with ZnO/H₂O and Zn²⁺ ions at equivalent doses, in a slightly more pronounced (up to a 25% decrease) and significant manner. However, these are relatively small decreases, and these data can therefore be considered consistent with those obtained in cell lines and during proteomic analysis: the few protein-level modifications induced by zinc are therefore insufficient to affect mitochondrial function and induce variations in the associated transmembrane potential. This seems consistent since the modified proteins are not involved in the mechanisms of electron and proton transfer at the mitochondrial membrane (respiratory chain), on which this potential strongly depends.

4.5. Effects of zinc on NO production:

To validate the observations from the proteomic analysis and targeted cell line assays, we also evaluated the effects of zinc on the ability of primary macrophages to produce NO, a pro-inflammatory mediator essential for the cellular response to exogenous stimulation by bacterial LPS. The cells were therefore exposed to cationic ZnO, ZnO/H₂O, and Zn, then

stimulated with LPS. The NO produced and secreted into the culture medium was then measured using the Griess method, under the same experimental conditions as with the cell lines. The results of this assay are shown in Figure 15.

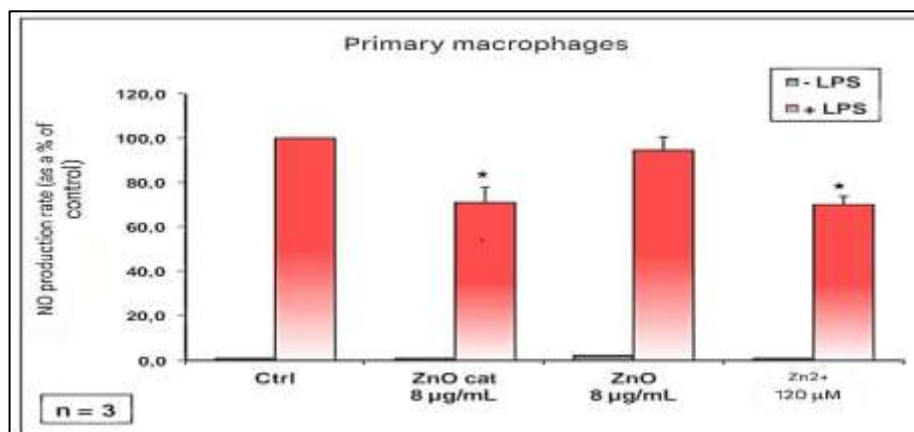


Figure 15: Effects of zinc on NO production by primary macrophages.

Cells were treated or untreated for 24 h at 37°C with NPs or ions at their LD20, then stimulated or unstimulated for 24 h at 37°C with LPS (1 µg/mL) in the presence of arginine (5 mM). NO secreted into the culture medium was measured by incubating the supernatant with an equivalent volume of Griess reagent for 30 minutes at room temperature. The absorbance of the complex formed was measured spectrophotometrically at 540 nm against a range of sodium nitrite (NaNO₂). The rate of NO production under the different conditions is expressed as a percentage of the stimulated control. Statistical significance according to the non-parametric Mann-Whitney U test: $p \leq 0.05$

These tests show that primary cells are once again more sensitive to NPs than cell lines: a significant decrease in NO production is observed after exposure to cationic ZnO. However, this decrease remains in the range of 25 to 30%, which is significantly less than the decrease observed with ions in cell lines, and no significant decrease is observed with ZnO/H₂O. It can be considered that the effects of zinc on the production of this inflammatory mediator are relatively moderate, and that the cells are able to compensate for them. Conversely, the effects of ions are significantly less pronounced than in cell lines, and of the same order of magnitude as those of cationic ZnO, at equivalent doses. This result is surprising, given the generally increased sensitivity of primary cells, but it is conceivable that the observed difference is related to physiological differences between primary cells and cell lines. The overall results obtained from proteomic analysis and targeted NO assay, both on cell lines and primary macrophages, suggest that zinc in nanoparticle form may have a very moderate effect on the production of inflammatory mediators. However, these effects may be primarily related to the ionic fraction resulting from the significant intracellular dissolution of these particles, with the effects of the ions being more pronounced and already documented in the literature (Pluth, M.D., et al., 2011).

4.6. Effects of Zinc on Metabolism: Pyruvate Assay:

As with the RAW 264.7 cell line, the effects of zinc on pyruvate production by primary macrophages were also evaluated to further confirm and definitively validate the proteomic analysis data. Cells were exposed to nanoparticles and ions, then lysed. The pyruvate produced was measured enzymatically, with the reaction leading to the formation of a colored compound proportional to the amount of pyruvate present in the sample. Due to time constraints and the limited quantity of biological material and enzymatic reagents available, this assay was performed only once. The preliminary results are shown in Figure 16.

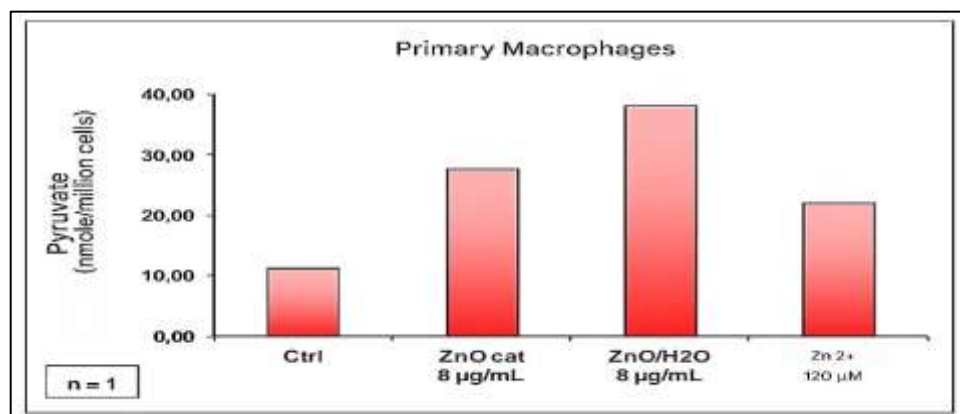


Figure 16: Effect of zinc on pyruvate production by primary macrophages.

Cells were exposed for 24 h at 37°C to NPs and ions at their respective LD20s, then harvested and lysed. The amount of pyruvate produced was determined enzymatically. The absorbance of the colored compound formed,

proportional to the pyruvate present in the sample, was measured at 560 nm. The amount of pyruvate was expressed as a function of the number of cells in the sample.

As in the RAW 264.7 cell line, pyruvate production is increased with both ZnO nanoparticles and with Zn ions, and according to the same trends: the increase is at least a factor of 2, and up to 4 in the case of ZnO/H₂O. These preliminary data are therefore perfectly consistent with those obtained from the RAW 264.7 cell line. All of these results confirm that zinc, regardless of its form (nanoparticle or ion), induces stress in cells, to which they respond primarily by over-activating their central metabolism. This increased pyruvate production can notably be used to meet the increased energy demands of cells, allowing them to compensate for the stress induced by zinc exposure.

5. Conclusion

In this study, two types of ZnO nanoparticles (NPs) were used: one obtained as a commercial dispersion, and the other as a powder whose dispersion in aqueous solution does not require a coating agent. Both types of NPs form aggregates of approximately 200 to 250 nm, which macrophages are able to internalize and dissolve via intracellular degradation pathways, down to particles of approximately 25 to 30 nm. These NPs then exhibit toxicity at relatively low doses (4 and 8 µg/mL), particularly compared to other types of metallic NPs (TiO₂). The proteomic approach has revealed some of the molecular mechanisms explaining

this toxicity In particular, we have shown that molecular effects are primarily observed with zinc in its ionic form (120 μM), whereas the effects of nanoparticles (NPs), at equivalent doses, are very limited. We note mainly an overactivation of central metabolism, allowing for compensation of the increased energy demands associated with stress, as well as some slight modifications of intracellular internalization and degradation pathways, reflecting an effort by cells to compensate for the induced effects, which they appear to be able to do. Indeed, zinc does not induce intense oxidative stress in cells, intracellular glutathione levels remain stable, and the effects at the mitochondrial level are limited, affecting only a few non-membrane proteins, and therefore not inducing variations in the associated potential. Similarly, phagocytic capacity and NO production are only slightly affected. These moderate effects are explained in particular by the fact that the corresponding modifications often involve minor altered forms of proteins, while the major forms remain unchanged. Furthermore, the effects observed here are not very specific, since several of the proteins identified in the different pathways are part of the classic "already seen" in proteomics. All of these results suggest that the toxicity of zinc at the molecular level is essentially linked to the numerous interactions of the ionic form with a large number of proteins and cellular components. These interactions appear to be compensated by cells, up to a certain, relatively low threshold, beyond which disruptions to normal protein function lead directly to cell death.

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Survey and Checklist for Ecological and Geographical Distribution of Urticaceae Family in Iraq

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Abstract:

The investigation aims to species identification cultivated in Iraq for the family Urticaceae then definite the locations geographical and the environment of species by examining nemours references and preserved specimens in Iraq herbarium. The research indicates that the genus *Urtica* (L.) on three species and the genus *Parietaria* (L.) contains four species. An environmental study of this family indicated its distribution between diverse locations from forest zones, valleys and mountains, Shaded wetlands and other areas, noting the spread of some species within the diversity of soils and rocks. The study of the ecological distribution of species revealed that the *Urtica* (L.) species is restricted to the mountainous region, upper plains, foothills regions and lower Mesopotamian region. The plant *Parietaria* (L.) is found in mountainous areas, upper plains, foothills regions and desert plateau regions. The *Parietaria* (L.) species belong to the family are characterized by widely distribution in ecological variation and regions and the species *P. rechingeri* (Chrtek) is uncommon in Iraq, where it was spread in only one governorate within the mountainous region.

Key words: *Ecological Distribution, Mountain Region, Upper Plains and Foothills Regions, Geographical Distribution.*



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Introduction

Urticaceae, the nettle family is a main family of the angiosperms flowering plants, comprising about 54 genera and more than 2000 species of herbs, shrubs, small trees, and a few vines, distributed primarily in tropical regions (3, 4). The family name comes from the genus *Urtica* (L.) and Stinging nettle is distributed nearly worldwide but is especially common in Europe, North America, North Africa, and parts of Asia (9). The Family Urticaceae is classified as follows: Kingdom: Plantae, Class: Magnoliopsida, Order: Rosales (10). In Iraq, this family comprised 40 genera and about 600 species, mostly in tropical and subtropical areas: 2 genera in Iraq (1, 5)

The Urticaceae family is characterized by erect or ascending stems, opposite or alternate leaves, Simple, Stipules generally existent, Flowers monoecious or diecious, arranged in a bracteate cyme which may be modified into a dense head by the reduction of axis and pedicels or reduced to a single flower, Inflorescence axillary in the upper leaf axis (15, 16). A review of the currently available literature shows numerous reports on the plant components found in many species of the Urticaceae family (8). Geographical differences and environmental factors are amongst the significant agents and features that affect the morphological construction for living organisms. Then, it is important to identify various ecological agents, such quality of the soil, humidity, light rigor, and the distribution for the plant species position, and to definite the possibility for these agents are related to the morphological characteristics for the various taxonomic orders. The significance for geographical distribution has emerged in some several classifications with restricted distribution for the species in a certain environmental region, making it singular methods utilized in the methods for isolating, and classifying species. (14). Otherwise, geographical guide demonstrates the origin, development, emigration, and distribute for the species, by giving similarities and variations in the morphological as well as anatomical characteristics for ranks classification at different stages are linked to the scope of distribution, so narrow or broad (11). Due to the scarcity in the Iraqi references that deal with datum about the family of Urticaceae, as well as the nonappearance of a recent elaborated study on the ecological distribution for species for this family and their geographical places in Iraq, this research was prepare.

Materials and methods

This research depended on the datum recorded on the dried specimen's identification card reserved in the herbarium of Iraqi. This information was installed for each sample identification card, which included all the information recorded on it; and

much of the information has been published by researchers, such as (17, 2, 12, 6 and 13). The Guest data was utilized and revised to demonstrate the distribution regions for the studied species on maps (6). Map shows spread locations for species plotted across the geographic regions listed below.

Table 1. Regions and phytodistricts of Iraq according to (7)

M	Mountain Region
MAM	Amadiya District
MRO	Rowanduz District
MSU	Sulaimaniya District
MJS	Jabal Sinjar District
F	Upper Plains and Foothills Regions
FUJ	Upper Jazira District
FNI	Nineveh District
FPF	Persian Foothills District
D	Desert Plateau Region
DLJ	Lower Jazira District
DWD	Western Desert District
L	Lower Mesopotamian Region
LCA	Central Alluvial Plain District
LSM	Southern Marsh District

Results and Discussion

The *Urtica* (L.) genus in Iraq includes three species, namely *U. urens* (L.) was found in the steppe and desert regions of Iraq such as Singar (FUJ/MJS), 10 km north of Mosul (FNI) 25 km north east of Baghdad and 7 km south west of kut within the (LCA) District, 5 km west of Amara and Qal'at Salih within the province of (LSM) in clay soil, its habitate waste places, along roadsides, in gardens; up to altitude 500 m, Its climate a mild to warm and the humidity should be moderate. While the species *U. pilulifera* (L.) spread in the lower forest zone and steppe region of Iraq, rare in the desert region, found on 10 km west of Aqra (MAM), 5 km south east of Shakh- Harir (MRO), Makhlat (FUJ/FNI), Hadhr (FUJ), Jabal Maklub (FNI) and 2 km north of Ana (DWD) District, in light clay soil, its habitate among limestone rocks, calcareous waste places; altitude 150-1000 m, the climate a temperate to warm and the humidity should be moderate as it cannot tolerate prolonged drought. The species *U. dioica* (L.) was spread in the forest zone of Iraq. This species was present on west for Mar Ya'qub, 5 km for north of Sharanish, Ardin, 20 km south west of Zawita and Sersang within the (MAM), 12 km. of Algurd Dagh, Algurd Dagh near Sarcal, 750 m of Rayat, Rost, Chiya- Mandue near Walash, north Kodo, around Germasur lake north east of Rania, Qandil range within the province of (MRO), Penjwin, south of Tawana in (MSU), in fertile clay, its habitate among damp rocks, between broken stones on newly made mountain road, in gardens, shady moist wasteland; altitude 950-1750 m. its climate is temperate to relatively cold and keep moist continuously. The other genus belong to Urticaceae family is *Parietaria* (L.) included four

species and it tolerates drought more than *Urtica* (L.) genus, which is *P. Judaica* (L.) distributed north west sector of the forest zone of Iraq, apparently rarer in the south east sector, found in Zinta gorge, north of Zakho 60 km south west of Sharanish, Severa Gaurik, Gali Zawita near Turkisk frontier, Matina, 35 km south of gorge, Amadiya, Sharifa, north of Mons Gara (Gara Dag) north east of Dohuk, Sersang, west of Shaikh Adil, 62 km south of Aqra within the province of (MAM), in (MRO) District found on 10 km of Gali Ali Beg, 23 km east of Rawanduz Gorge, Khalifan, east of Bekhal, Botin, near Rawanduz, Jandian, 10 km north of Haji Umran, roadsides of Sakri Sakran, Baisar, Qandil rang, Wartanorth west by west of Rania, west slope of Jabal Avronman in (MSU), Tal Afar within in (FUJ) District, in clay soil, its habitate shady rocks; rocks near waterfalls; on limestone and metamorphic rock; sometime in oak forest; altitude 300-500m, the climate and humidity are characterized by being moderate. While the species *P.rechengri* (Chrtek) was distinguished its presence in only one locality in the north west sector of the forest zone of Iraq. Found on Jabal Khantur near Sharanish in (MAM) District, in clay soil, its habitate rock crevices on mountain; on calcareous rock; altitude 1200m, its climate mild to relatively dry and the humidity was moderate. The species *P. lusitanica* (L.) was spread in the forest zone of Iraq. The species was found on north east of Zakho, Sersang, 15 km south of Aqra in (MAM), east of Pushtashan in (MRO), in (MSU) District it found in Sulaimaniya, north west of Khormal, 35 km north of Penjwin, Tawila, south of Humaidat, above Mosulin (FUJ) and in (FNI) found in Makhlat, south of Bashiqa, in rocky soil, its habitate shady rocks; gypsum outcrop on riverside bluff; north stream in shade of oak trees; altitude 250-850 m, a mild to warm climate and moderate humidity are preferred. While the species *P. alsinifolia* (Del.) found in the steppe and desert regions of Iraq. Present in Jarmo (MSU), Rawa (FUJ), east of Makhlat (FNI), 10 km east of Mandali, Koma Sang, north of Persian frontier, east of Zurbatiya, Buskaya in (FPF) District, north river Euphrates (DLJ), Haditha, 41 and 95 km north east of Rutba, 15 km south of Rutba within the (DWD), in rocky soil, its habitate shady places under overhanging rocks, crannies in limestone cliffs, sandy gravel hillsides; altitude 150-650 m, However, the climate and humidity are similar to the species *P. lusitanica* (L.).

Conclusion

The present research has shown that all genus for Urticaceae family is widespread throughout Iraq, However, it is more predominant in the northern and middle areas for Iraq, while it is minimal prevalent in southern Iraq, and this shows that it is not appropriate to the environmental conditions in southern Iraq, with high temperatures and low rainfall. It was observed that the genus *Parietaria* (L.) more tolerates drought than *Urtica* (L.) genus.

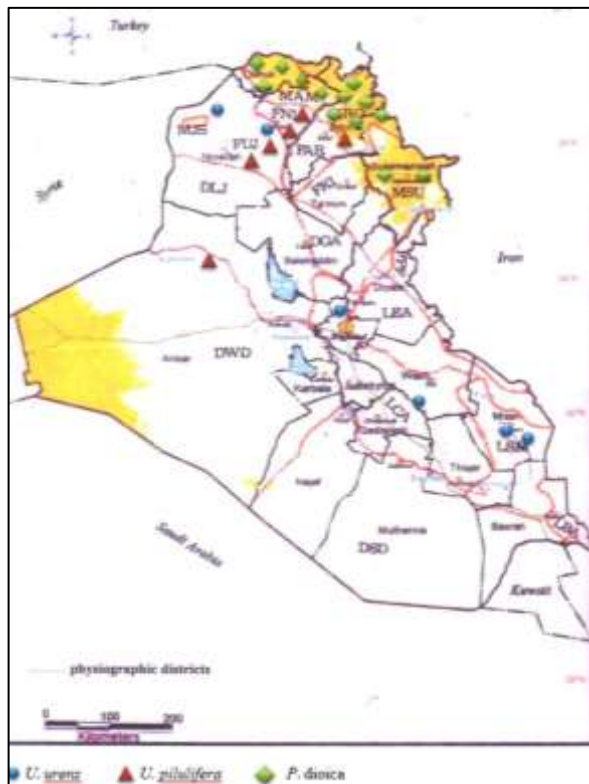


Figure 1: Geographical distribution of genus *Urtica* (L.)

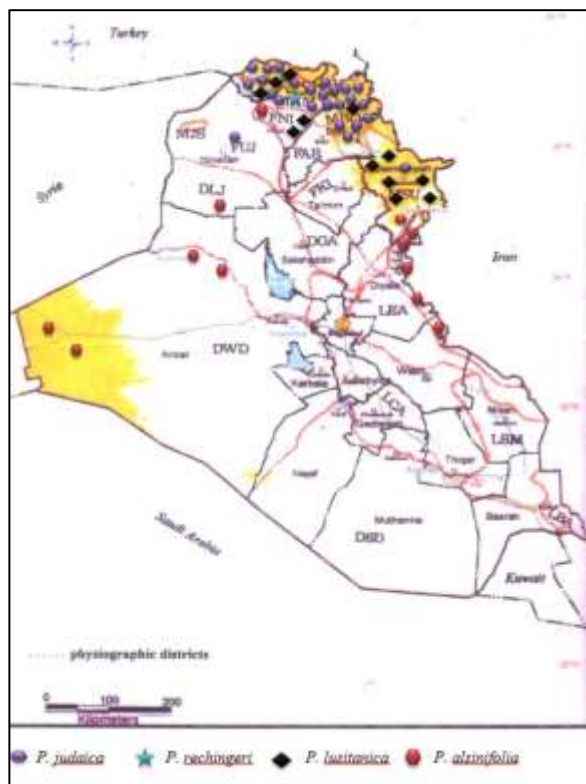


Figure 2: Geographical distribution of genus *Parietaria* (L.)

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The Behavior of Ring Footings on Sandy Soil under Eccentric Load

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Abstract:

A number of works have been carried out for the evaluation of the ultimate bearing capacity of shallow foundation embedded in sand and subjected to centric load. Few experimental studies have been made on the calculation of bearing capacity of shallow foundation on sand under eccentric loading. However these studies are for strip footings. This paper presents the behavior of ring footing under eccentric loading on sand. The model tests have been conducted using ring footing with ring radii ratio (D_i/D_o) = 0.2, 0.4, 0.6 on cohesionless soil with different relative densities under eccentric load with different ratio of eccentricity e/D_o = 0, 0.15, 0.3 and 0.45. This paper used dense, medium, and loose sand with soil unit weight are 18.3, 16 and 14.6 kN/m³ for three relative density equal to 80%, 50%, and 30% respectively. Parametric studies have been made to find the impact of eccentricity ratio, ring radii ratio and density of soil on bearing capacity of the ring foundation. The experiments conducted found multiple ring foundation types resulted in varying capacities for each foundation. The highest capacity recorded by different foundation types was $n=0.4$. Conversely, the lower capacities were found in foundations $n=0.2$ and $n=0.6$. There is also a strong correlation between the capacity and the density of the soil, in that regardless of the density of the soil, as e/D_o (the ratio of load eccentricity to the diameter of the footing) increased, the ultimate capacity of the $n=0.4$ ring footing decreased. The ultimate capacity of an $n=0.4$ ring footing experiences a 29%, 61% and 65% reduction in bearing capacity if subjected to e/D_o ratios of 0, 0.15, and 0.3 respectively, with a 30% relative density of the soil.

Keywords: *Ratio Of Eccentricity; Radii Ratio; Bearing Capacity; Ring Footing; Experimental Study.*



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1. Introduction

A ring foundation is a common annular structure which is more applicable for certain kinds of structures like silos, cooling towers and oil depots, transport and high-level tanks etc. There is no weak corner in circular and ring footings, hence stresses do not concentrate at any point of foundation. From the economy point of view whenever feasible, a ring foundation is preferred to circular one as it takes lesser quantity for construction. Kern distance also increases for ring foundation in comparison with circular foundation. Stratum and statistical procedures are less common in evaluating ring foundation bearing capacity, as well as settlement in comparison with strip, square, rectangular and circular foundations (Kumar, 2015; Huda, 2025).

Several authors have carried out analytical as well as numerical studies on eccentrically loaded footing resting on unreinforced and reinforced soil. Zhu (1998) performed numerical analysis and physical modelling and subsequent of the bearing capacity for ring foundations on sand to obtain the vertical load-carrying capability of ring footing over dense sand. It was noted that the lowest bearing capacity for eccentrically loaded ring foundations is reduced with increasing load eccentricity.

Mehrjardi (2008) studied the capacity for bearing with a continuous footing over sand; a comparison was made between the ring footing and circular footing types, as well as how each is designed. Sawwaf & Nazir (2012) performed an experimental investigation on eccentrically loaded, model circular footings supported by compacted sand with a well compacted layer of loose sand underlying the compacted layer. The BCR of the ring footings significantly increases with the use of soil reinforcement injection, and continues to increase with the addition of geogrid reinforcement layers for either sand density.

Hussein and Albusoda (2013) investigated the behaviour of a square footing placed on Reinforced Dunes Sandy soil over Gypseous via Eccentric Loading. Their studies indicated that after the improvement of Gypseous Soil the bearing capacity of the foundation increased by 2.5-3.0. Dhar et al.'s (2013) study looked at the load bearing capacity of a rigid footing under Eccentric and Inclined Loading and concluded that with the increase of load eccentricity in either Inclined or Vertical loading; the load bearing of the foundation would decrease regardless of eccentricity. Also, they noted that as eccentricity increase in load, the bearing capacity of the footing decreases when the degree of Inclination is increased.

Algin (2014) offers a thoughtful exploration of how eccentrically loaded rectangular footings resting on sand deposits undergo elastic settlement, grounding the inquiry in a detailed study that bridges theoretical insight with practical engineering concerns. For the determination of vertical elastic settlements under eccentric foundations, a closed shape analytical resolving (with correlated factors of influence) was carried out. In the hypothetical cases where this layer of dirt is infinitely deep, we derive the equations in more detail.

2. Bearing capacity and load eccentricity

There are two ways of considering the bearing capacity of footings with eccentric loads when considering the foundation engineering sector. These are effective area and reduction factor methods. The effective area method relies upon Meyerhof's assumption for the dimension reduction of the footing from the original size of the footing; so as to provide a uniform pressure throughout the footing and onto the supporting soil through what he called an effective area (Meyerhof, 1953). The value of the uniform pressure imposed by this new effective area was called an equivalent uniform bearing pressure; however, it does not necessarily obey the actual distribution of the load across the footprint of the footing. The value of the equivalent bearing pressure is multiplied with the value of the effective area to provide a maximum ultimate load the footing can bear (Loukidis, 2008). There have been numerous literature reviews regarding the use of this method (Hansen, 1970). The effective area method has been generally accepted as reliable for the majority of applications for strip, rectangular, and circular footings, although this method provides conservative estimates of the ultimate load that can be supported by the footings. (Vesic, 1970).

The effective area method is applicable for the footings where the geometry of the equivalent area can be simplified as a rectangle or a circle, whose bearing capacity equations are available. Fig. 1 indicates the concept of effective area method for a rectangular footing with length (L) and width (B), a circular footing with diameter (D) and a ring footing with outer (D_o) and inner (D_i) diameters. As shown in parts a and b of Fig. 1, the hatched area over which, an equivalent uniform pressure is applied, can be considered as an equivalent rectangle (with effective width B_o) or circle (with effective diameter D_o) and then, the bearing capacity of the equivalent footing (with the area A_o) is calculated. For ring footings, according to Fig. 1c, it is not possible to find a homothetic surface lying in the hatched area and thus, the effective area method is not suitable for ring footings.

As for the second approach known as the reduction factor method, the bearing capacity of eccentrically-loaded footing (q_{ult}) is calculated directly by multiplying a reduction factor (Rf) by the bearing capacity of the footing under vertical load $q_{ult (e=0)}$:

$$q_{ult} = Rf \cdot q_{ult (e=0)} \quad \dots (1)$$

In this method, the area of the footing is not changed and thus, the ultimate load capacity with eccentricity P_{ult} can be calculated similarly as $P_{ult} = Rf \cdot P_{ult (e=0)}$ where $P_{ult (e=0)}$ stands for the ultimate load of the footing with centric load. This method was initially introduced by Meyerhof (1953) and now it is used for strip footings. This method was investigated experimentally by Aiban and Znidarcic (1991) and then, it has been extended to consider footing embedment as well as soil improvement effect by reinforcement layers (Seyedi, 2016). In this method, no assumption is made for the eccentrically-loaded footing area and the effect of load eccentricity and generally, the failure mechanism is considered in the reduction factor. Since no simplified shape of footing (such as rectangle or circle) is required, this method is suitable for studying the bearing capacity of ring footings with

eccentric load. The ultimate bearing capacity of an eccentrically-loaded ring footing (q_{ult}) can be expressed in a similar form of Terzaghi's equation (Patraa et al., 2006) as follows:

$$q_{ult} = C N_c(e) + q_0 N_q(e) + 0.5 D_o \gamma N_\gamma \quad \dots (2)$$

Where c is the soil cohesion, q_0 is the surcharge over the surface, γ is the soil unit weight and D_o is the outer ring diameter. N_c , N_q and N_γ are bearing capacity factors in which the shape factors are included. The subscript (e) denotes the consideration of load eccentricity. In the case of a ring footing embedded on the surface of a cohesionless soil, the bearing capacity of a ring footing is simplified into:

$$q_{ult} = 0.5 D_o \gamma N_\gamma \quad \dots (3)$$

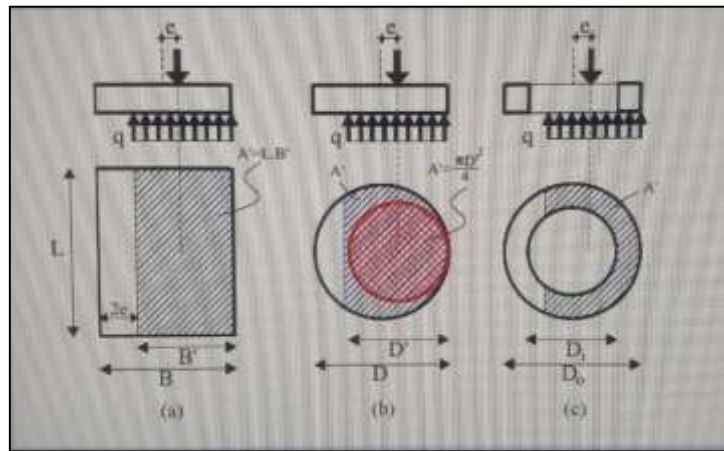


Figure1: Concept of effective area method in order to consider load eccentricity for: (a) rectangular footing; (b) circular footing; (c) ring footing.

3. Methodology

The main objective of experiment is to study the behavior of eccentrically loaded small-scale ring footings resting on sandy soil.

3.1. Test Material and their Properties:

3.1.1 Test sand:

For the model tests, cohesionless, washed, dried, and sorted by particle size was used as the foundation material. The distribution of grain size for the soil, determined through standard sieve analysis, is listed in figure 2. The properties of sandy soil such as Bulk density, specific gravity, coefficient of uniformity, etc. are shown in table1.

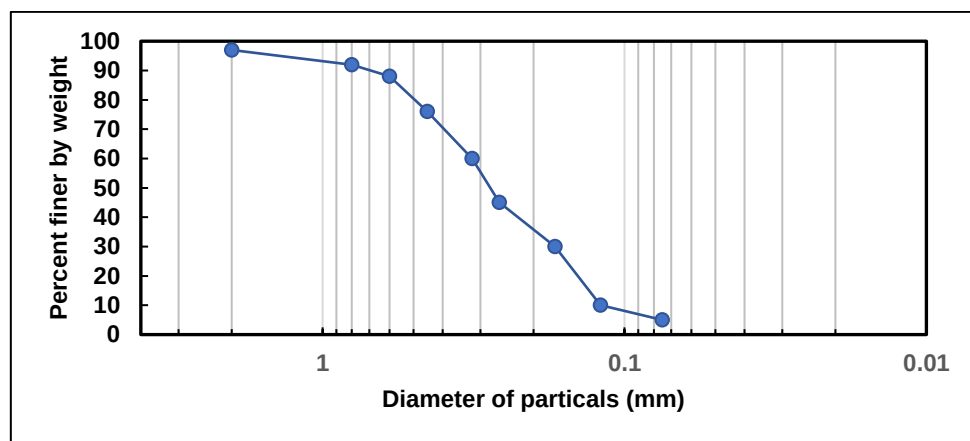


Figure 2: Distribution of grain size curves for soil.

Table 1: The properties of soil.

Sr. No.	Properties	Value
1	Bulk Density (kN/m^3)	16
2	Specific Gravity	2.7
3	γ_{max}	18.3
4	γ_{min}	14.6
5	$(\phi \text{ peak})^\circ$	47
6	$(\phi \text{ critical})^\circ$	28
7	Coefficient of Uniformity C_u	3.6
8	Coefficient of Curvature C_c	4.5
9	Effective Size D_{10}	0.14

3.1.2 Ring footing model:

The model ring was fabricated by using mild steel plate. Model footing consists of ring footing of 80 mm outer diameter and different inner diameters was considered. The inner diameters 16 mm, 32 mm, 48 mm were considered. The details of ring models are shown in figure 3.

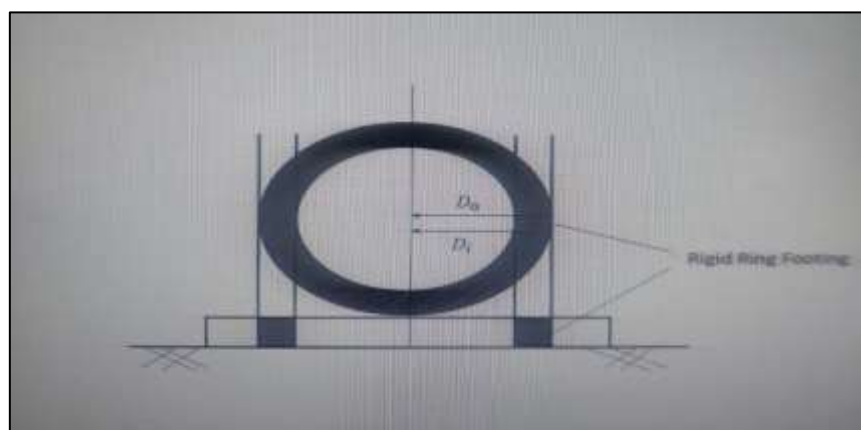


Figure 3: Details of ring model.

3.2. Procedure for Test Setup:

The detailed test procedure adopted for experimental investigation is explained below-

3.2.1. Preparation soil bed:

The soil bed was carefully prepared inside the container by forming five distinct layers, each uniformly compacted to a thickness of 100 millimeters. A predetermined amount of soil was placed for each layer and then compacted using vibratory equipment. This process enabled the attainment of target dry unit weights of 14.6, 16.0, and 18.3 kN/m³, which correspond to 30%, 50%, and 80% relative densities, respectively, depending on the experimental conditions. The container itself measured 0.5 m in length, 0.5 m in width, and 0.6 m in depth.

3.2.2. Model Plate Load Test Procedure:

For the experimental work, the model plate load tests were conducted on sand as per IS 15284 (Part 1): 2003 to evaluate the bearing capacity and settlement. After preparation of sand bed, the model footing was placed at the center of the tank. Then two dial gauges placed on the flanges of the footing. The load was applied on the footing with the help of screw jack in increments. Each load increment was approximately equal to 1/5th of the expected ultimate load. The load transferred to the footing was measured through proving ring placed between the footing and screw jack. Footing settlements was measured through two dial gauges placed on either side of the center line of the footing. The footing settlement was reported as the average value of the readings taken at the two different points. The experimental test setup is shown in Figure 4.

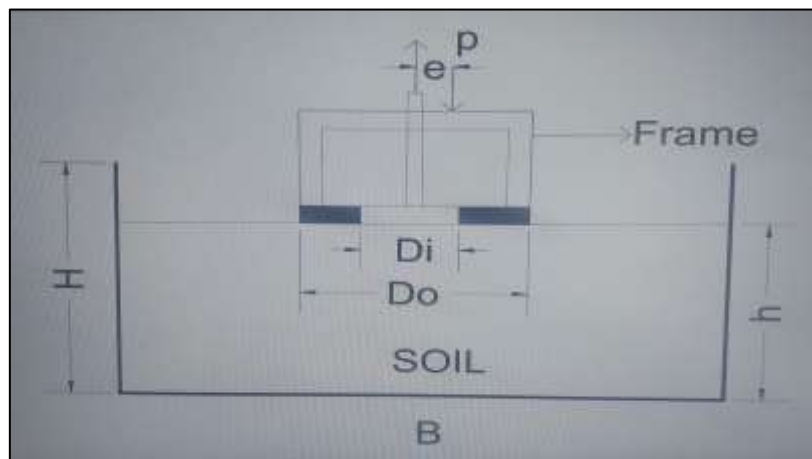


Figure 4: Equipment Setup

4. Experimental Results

4.1. Ring footing model under centric loading:

The test was conducted on ring footing of different ring radii ratios (D_i/D_o) 0.2, 0.4, 0.6 with different percentage of relative densities of 30%, 50%, and 80% at a ratio of eccentricity $e/D_o = 0$ on sand. The corresponding load settlement graph is drawn. The load settlement graph for ring footing of $D_i/D_o=0.2$, 0.4 and 0.6 with relative density equal to 30%, 50% and

80% under centric loads as shown in figure 5. Table 2 listed the ultimate bearing capacity. The value of ultimate bearing capacity estimated at settlement equal to 5%D0 (Huda, 2025).

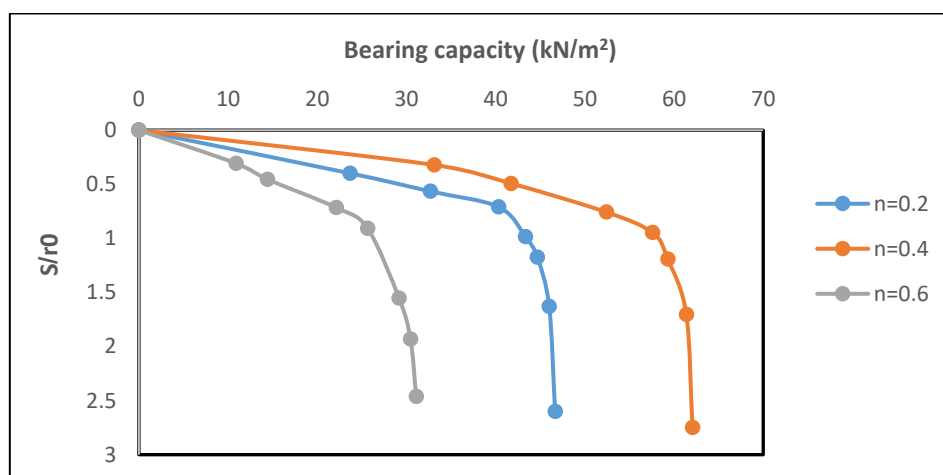


Figure 5 a: bearing capacity-settlement / external radius curve of ring footing with different radii ratio on sand at Dr=30%.

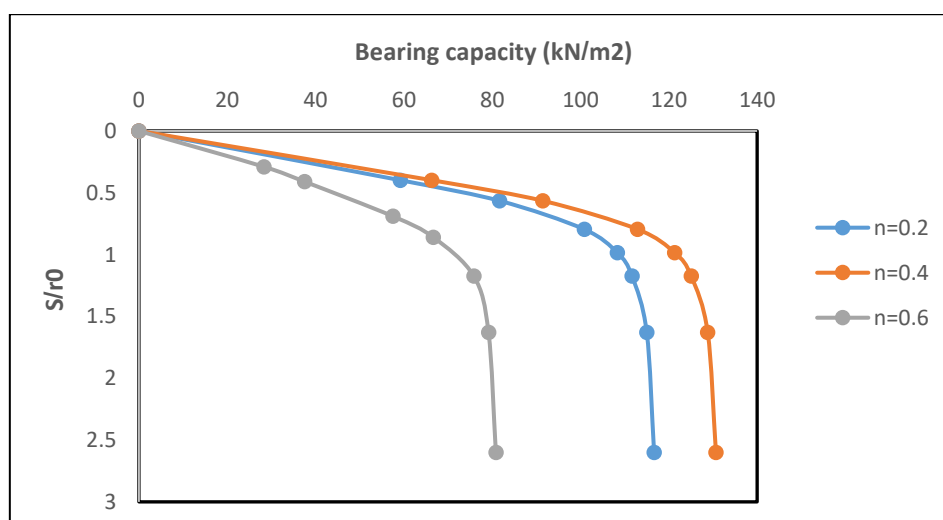


Figure 5 b: bearing capacity-settlement / external radius curve of ring footing with different radii ratio on sand at Dr=50%.

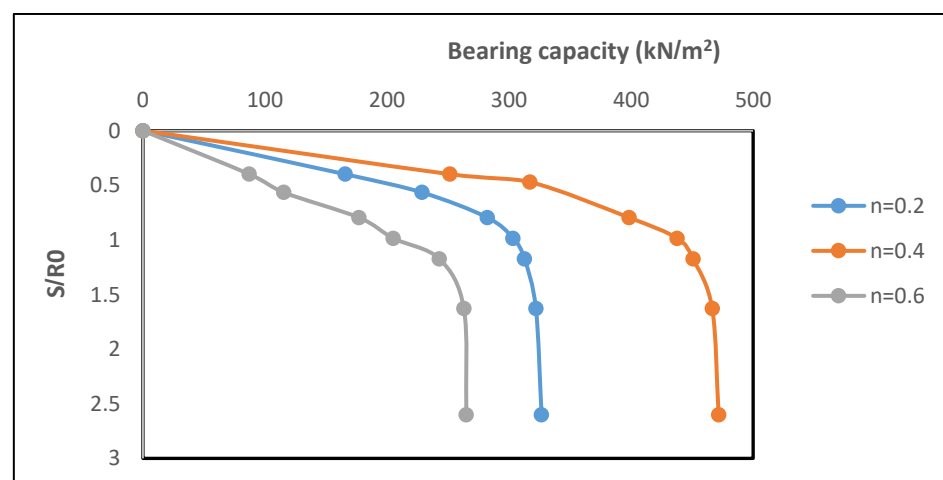


Figure 5 c: bearing capacity-settlement / external radius curve of ring footing with different radii ratio on sand at Dr=80%.

Table 2: Ultimate bearing capacity of ring footings with different ratio of r_i/r_o .

Dr %	r_i/r_o	Bearing capacity (kN/m²)
30%	0.2	46
	0.4	55
	0.6	23
50%	0.2	108
	0.4	121
	0.6	66
80%	0.2	303
	0.4	437
	0.6	205

The results indicate that both the soil's density and the radii ratio (n) significantly influence the behavior of a ring footing. An increase in relative density enhances the bearing capacity, reflecting greater soil strength and improved resistance to shear failure.

The bearing capacity is also substantially affected by the radii ratio, defined as the ratio of the internal ring radius (r_i) to the external ring radius (r_o). Experimental investigations on model footings reveal that ring foundations with a radii ratio of $n = 0.4$ exhibit superior performance, yielding higher bearing capacity values. In contrast, foundations with ratios of $n = 0.2$ and $n = 0.6$ show reduced bearing capacity compared to the $n = 0.4$ case. This improvement at $n = 0.4$ can be attributed to the confinement of the soil within the ring, which undergoes significant compression and effectively acts as an integral part of the footing. This results in a combined system with a total effective area greater than the contact area of other ring footings, thereby contributing to the enhanced bearing capacity.

4.2. Ring footing model under eccentric loading:

The test was conducted on Ring footing with optimum state of ring radii ratios (D_i/D_o) = 0.4 with different percentage of relative densities of 30%, 50%, and 80% under eccentric load with different ratio of eccentricity $e/D_o = 0, 0.15, 0.3$ and 0.45 on sand. The load settlement graph for ring footing of $D_i/D_o=0.4$ with relative density equal to 30%, 50% and 80% under eccentric loads with $e/D_o = 0, 0.15, 0.3$ as shown in figure 6 and table 3 summarized the ultimate bearing capacity of ring footings with different ratio of e/D_o .

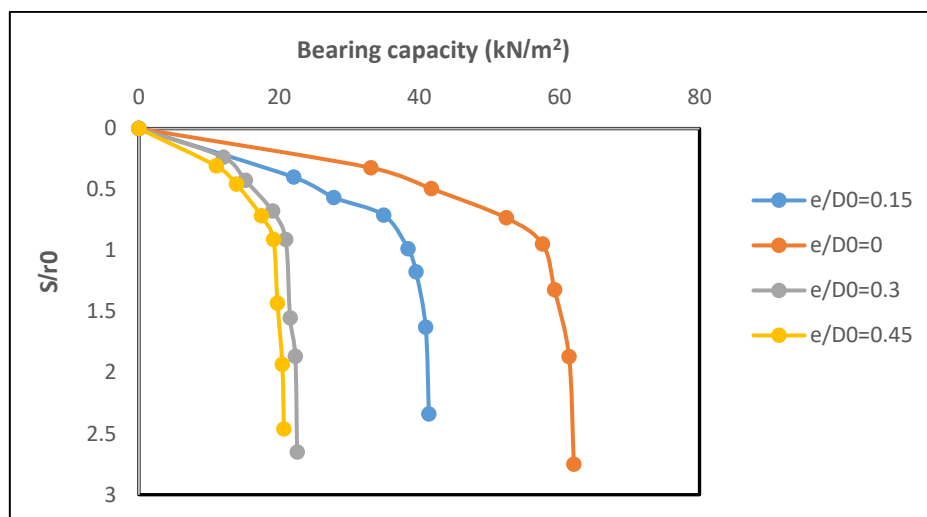


Figure 6 a: bearing capacity-settlement / external radius curve of different ratio of eccentricity for ring footing on sand with $Dr=30\%$.

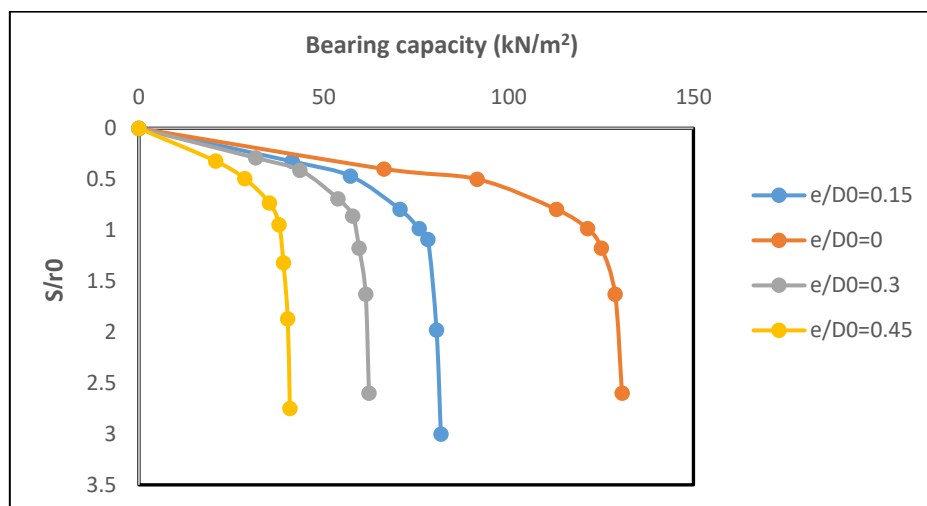


Figure 6 b: bearing capacity-settlement / external radius curve of different ratio of eccentricity for ring footing on sand with $Dr=50\%$.

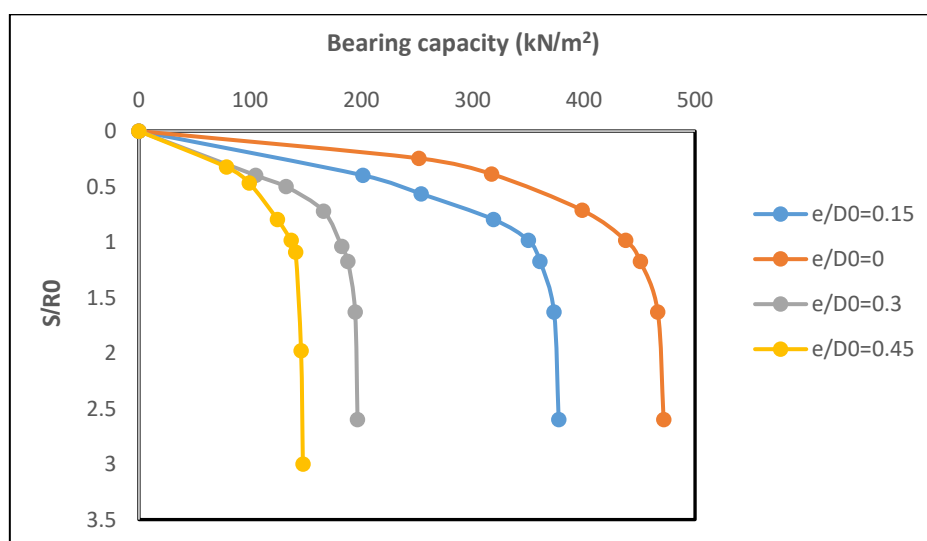


Figure 6 c: bearing capacity-settlement / external radius curve of different ratio of eccentricity for ring footing on sand with $Dr=80\%$.

Table 3: Bearing capacity of ring footings with different ratio of e/D_o .

Dr %	e/D_o	Bearing capacity (kN/m²)
30%	0	55
	0.15	39
	0.3	21
	0.45	19
50%	0	121
	0.15	76
	0.3	58
	0.45	38
80%		437
	0.15	350
	0.3	182
	0.45	137

In this study the load eccentricity is applied at the right side of the footings. When the footing is loaded vertically, the stress disturbance under the footing region is less than the cases with load eccentricity. This means that the load eccentricity causes more stress concentration under the footing surface and especially the edges. As the e/D_o increases, bigger stress concentration exists under the right side of the footing. The magnitude of stress concentration is larger for big eccentricity ($e/D_o = 0.45$), because the ring footings with $e/D_o = 0.15$ still have contact with the ground surface while the ring footings with $e/D_o = 0.45$ have rotated and one side (left side) of the footings has been uplifted. This explains the decreasing in the value of bearing capacity of ring footing with increasing the ratio of eccentricity e/D_o .

5. Concluding

In this paper, the bearing capacity of ring footings under centric and eccentric loading condition is investigated for the ring footings resting on the surface of cohesionless soil. This paper uses experimental tests to examine the behavior of ring footing placed on the sandy soil surface with different relative density, the ratio of eccentricity and radii ratio and following notes are observed:

- The bearing capacity of ring footing is found to be maximum at ring radii ratio (D_i/D_o) of 0.4.
- The bearing capacity of ring footing rises with increasing the relative density of soil under centric and eccentric loading.
- As eccentricity increases bearing capacity of ring footing decreases with different value of relative density of sandy soil. The reduction ratios in the bearing capacity of ring footing under eccentric loads with $e/D_o = 0, 0.15, 0.3$ at 30% relative density are 29%, 61% and 65% respectively.

This study concerns the use of the bearing capacity of ring foundation under centric and eccentric vertical loads located on cohesionless soil only.

Glossary

e = Eccentricity

P = Load intensity

$D_r\%$ = relative density of soil

h = Depth of soil

B = Tank's width

H = Tank's height

n = ratio of ring radii

D_i = inner ring diameter

D_o = outer ring diameter

q_u = ultimate bearing capacity

N_q, N_γ = bearing capacity factors

ϕ = frictional angle

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Fabrication of Ag-, Sn-, and Zn-Doped Titanium Dioxide Nanotubes by Pulsed Laser Deposition

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Abstract:

This study focuses on how the presence of Ag, Sn, and Zn affects crystallinity, surface structure, and potential charge-transfer behavior in TiO₂ nanotubes. In general, metal doping of TiO₂ nanotubes is used to tune the band gap, reduce electron-hole recombination, and enhance performance in photocatalysis and related optoelectronic uses. Pulsed laser deposition, a technique known for producing high-quality thin films with controlled composition and structure. Doping enhances the structural, optical, and functional characteristics of TiO₂ for cutting-edge applications while altering the nanotube shape.

The study focuses on the effects of Ag, Sn, and Zn on TiO₂ nanotube crystallinity, surface structure, and potential charge-transfer behavior. TiO₂ nanotubes are typically doped with metals to adjust the band gap, lower electron-hole recombination, and improve photocatalysis and associated optoelectronic applications.

TNTs were assessed using a variety of characterisation methods. Energy dispersion spectroscopy (EDS), X-ray diffraction (XRD), and Atomic Force microscopy (AFM) data show that morphology of Ag, Sn, and Zn was produced.

Overall, the fabrication strategy combines the advantages of TiO₂ nanotube architecture with the property-modifying effects of metal dopants, making the material promising for sensors, photocatalysis, and energy-conversion devices. The development of gas sensors focused on the unhealthy gas hydrogen sulfide (H₂S). The sensor composed of these nanotubes has a high sensitivity at various temperatures. The H₂S detection properties were assessed at temperatures between room temperature and 300°C. The gas sensor TNTs that were manufactured at 30 V for H₂S gas detecting have the maximum sensitivity and the fastest reaction time at room temperature, according to the results.

Keywords: *TiO₂ Nanotubes, Hydrogen Sulfide (H₂S), Energy Dispersion Spectroscopy (EDS), X-ray Diffraction (XRD), and Field Emission Scanning Electron Microscopy (FESEM).*



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1. Introduction

Titanium dioxide (TiO_2) has garnered significant attention due to its exceptional properties, including high chemical stability, biocompatibility, photoactivity, and a wide band gap[1]. These characteristics make it appropriate for a wide range of uses, including biomedical devices, gas sensors, solar cells, and photocatalysis. Although pure TiO_2 shows encouraging properties, its comparatively poor visible light absorption efficiency restricts its wider usage. By altering its electrical structure, increasing charge carrier separation, and extending light absorption into the visible spectrum, doping TiO_2 with metal ions provides a practical method to improve its performance. Among various deposition techniques, pulse laser deposition (PLD) stands out as a versatile method for fabricating high-quality, doped TiO_2 nanostructures, particularly nanotubes (TNTs). The fundamentals of PLD, its use in the synthesis of metal-doped TNTs, and the ensuing effects on the material's characteristics are all examined in this work [2].

After the target material is ablated by the laser energy, a plasma plume made up of ions, electrons, and neutral atoms is produced. The ablated material deposits as this plume spreads in the direction of a substrate at a certain distance, creating a thin coating. PLD's primary benefit is its ability to precisely transfer the target material's complicated composition and stoichiometry to the deposited film. In order to achieve regulated doping in TiO_2 , this is essential [3,4].

To produce a highly ordered array of TNTs on a titanium foil substrate, an anodization technique is first used. In order to enhance the production of tubular structures, this process depends on electrochemical oxidation in an appropriate electrolyte, usually including fluoride ions. The anodization parameters, including voltage, electrolyte content, and temperature, have a significant impact on the morphology and dimensions of the TNTs (length, diameter, and wall thickness). The TNTs are moved to a PLD system after anodization [5].

Usually, the target material is a carefully blended mixture of TiO_2 and the required metal dopant that is compressed into a pellet. Controlling the ablation process and the ensuing film growth depends heavily on the laser's parameters, which include wavelength, pulse duration, repetition rate, and fluence. Nitrogen, fluorine, iron, copper, and noble metals like gold and platinum are frequently used to dope TiO_2 . The final TNTs' optical and electrical characteristics are influenced by the dopant selection [6-8]. Doping with transition metals, such as iron, can result in defects and trap states that affect photocatalytic performance and charge carrier recombination. By encouraging surface plasmon resonance, noble metal doping can increase light absorption and boost the effectiveness of charge separation [10,11]. Precise control over the dopant concentration is made possible by the PLD process, which results in adjustable material characteristics. The PLD chamber's high vacuum environment reduces contamination and guarantees excellent film purity [13]. Additionally, the huge surface area of the TNT morphology—which is inherited from the anodization step—is advantageous for applications like photocatalysis that demand high surface reactivity [14]. Research has shown

that PLD-fabricated metal-doped TNTs have far higher photocatalytic activity than both pristine TiO_2 and other doped TiO_2 films made using different techniques [15–17].

In summary, pulsed laser deposition provides a potent and adaptable method for creating metal-doped titanium dioxide nanotubes. Researchers can customize the electronic and optical characteristics of TiO_2 to satisfy the particular requirements of diverse applications by combining anodization for TNT formation with PLD for controlled dopant incorporation. Researchers can modify the electrical and optical characteristics of TiO_2 to satisfy the unique requirements of different applications by combining anodization for TNT production with PLD for controlled dopant inclusion. Continued advancements in PLD techniques and target material design promise to further enhance the performance and broaden the applicability of metal-doped TNTs in diverse fields[18-21].

2. Experimental

2.1.Laser System:

Nd: YAG laser (Hua Fei Tong Da Technology –Diamond -288 Pattern EPLS) with different peak powers was used in the process of plasma creation under Vacuum[22]. The main technical parameters are:

- Q-switched Nd: YAG laser first harmonic generation laser type with λ (1064) nm.
- The number of pulse shots are (100 , 300 and 500).
- The target is around 10 cm away from the laser.

The laser energy can be increased in steps of 20 mJ, the laser spot's size may be adjusted by varying the distance between the laser lens and the target, and the number of pulses can be varied. However, the laser pulse duration is fixed at 9 ns. It is important to note that the temperature of the laser device must be monitored and controlled; ideally, the operating temperature should not be higher than 37 °C to prevent damage and malfunction of the internal active medium YAG crystal[23].

As was previously indicated, the laser is exiting through the gun hand piece. We must be aware that the device's pulse count ranges from 0 to 999999, after which the laser must be reset to get it ready to function once again (reset pulse counter). It should be mentioned that in order to maintain the water's purity and cooling effectiveness, it is nearly essential to replace the cooling water once a month[24,25].

2.2 PLD Technique and Fundamentals for the PLD System General Operation:

In order to oxidize target materials made of Ag, Sn, and Zn metals, PLD is often carried out inside a vacuum chamber at a pressure of 2.5×10^{-2} mbar. Focused laser beam Q-switching Nd: YAG enters the vacuum chamber through a Pyrex glass window and strikes the target's surface at an angle of 45° to its normal [26].

The substrate is placed in front of the target with its surface parallel to the target surface as shown in Figure (1 a,b), and there must be sufficient distance between the target and the substrate for the purpose of the substrate holder does not preclude the incident laser beam.

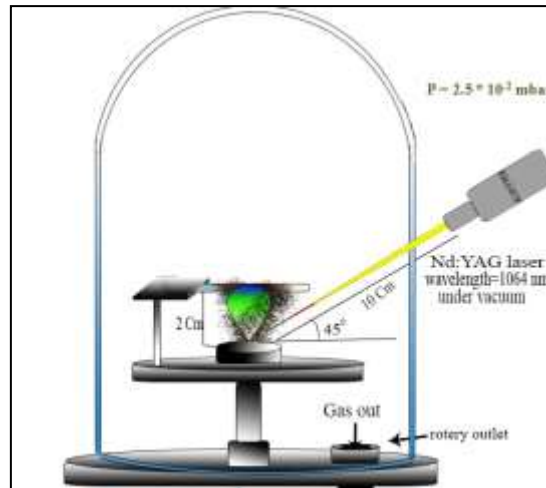


Fig. 1a . Schematic diagram of PLD setup with $\lambda=1064\text{nm}$.



Fig. 1b. Photograph of the PLD setup.

The vacuum chamber has a cylindrical form, and its design has to be simple and unrestricted. The evacuation system, pressure monitoring, target, substrate, laser beam control, and ports are only a few of the ports on the vacuum chamber. Additionally, it is crucial to consider the following factors while creating a vacuum chamber[27]:

1. The arrangement of the parts in the system PLD chamber shouldn't obstruct the laser beam's passage.
2. Since the target and substrate will alter the amount of pulses, access to them must be straightforward and simple.
3. The target-substrate distance needs to be flexible, meaning it may be changed.
4. Deposition in the laser window has to be minimized. Researchers occasionally modify the PLD process to make thin films of the highest quality. These adjustments include rotating the target, heating the substrate, and adjusting the substrate's location in relation to the target.

As seen in Fig. (2), a schematic graphic illustrates the experimental work in PLD.

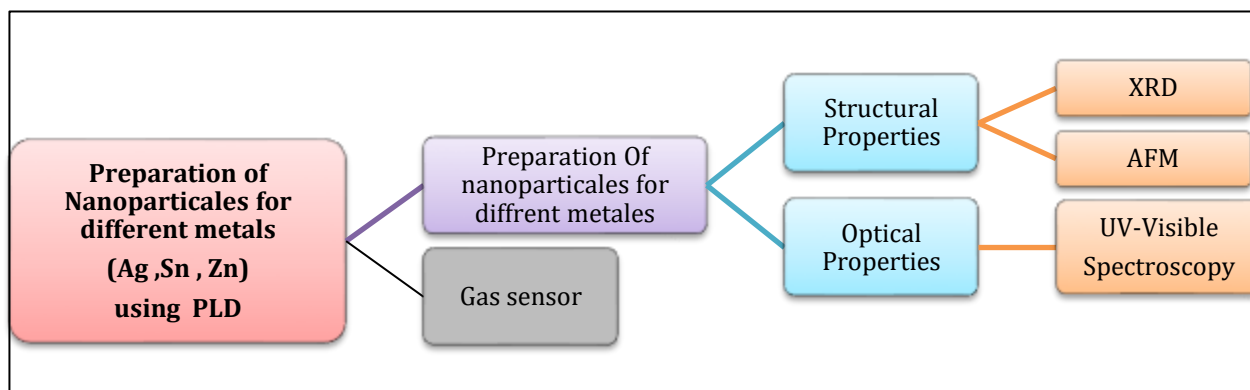


Fig. 2. A schematic diagram illustrates the experimental work in PLD

2.3 Laser Focusing:

A convex spherical quartz lens with a focal length of 10 cm was used to focus the Nd:YAG laser on the target surface, and the lens was positioned within the vacuum chamber. When the laser strikes the target material's surface, this laser focussing is required to surpass the ablation threshold energy density[28].

2.4 Laser Beam- Lens- Target Alignment:

In this experiment, the laser beam was manually aligned with the target's surface. Since the Nd:YAG laser is seen as a visible red hue, the calibration and alignment have been adjusted using a visible laser beam that serves as a guide.

The plume size of the laser-induced plasma is extremely tiny. A few millimeters of variation in the lens to surface distance can have an impact on the analysis intensity. For the system to be accurate and precise, it is crucial to maintain the distance constant throughout the measurements. We utilized a lens with a focal length of 10 cm in this work. Although a shorter focal length lens has a lower depth of focus, it might result in a narrower beam waist and, thus, greater breakdown[29].

2.5 Target Materials:

The metals (Silver (Ag), Tin (Sn), and Zinc (Zn)) of high purity with average sizes (2 cm x 2 cm x 0.3 cm) are used as targets during the experiments. The physical properties of these targets are listed in Table 1.[30-32].

Table 1 : properties of the target materials

Metal	Silver (Ag)	Tin (Sn)	Zinc (Zn)
Atomic No.	47	50	30
Melting point (K)	1234.93	505.08	692.68
Boiling point (K)	2435	2875	1180
Density (gm cm ⁻³)	10.503	7.289	7.140

3. Result and Discussion

3.1. Structural properties:

The main purpose of this section is to test the type of structure for **Ag, Sn, and Zn** metals prepared by the PLD technique related to this research and to ensure that the structure will not be changed after deposition. Laser pulse energy and laser wavelength have been studied. The structure of the thin films was measured by X-ray, and also The nanoparticle size was determined using the Scherrer equation, AFM. The formation of thin films using PLD is the process that depends on the physical parameters such as density, kinetic and potential energies, ionization degree, and type of the condensing particles, in addition to the temperature and physicochemical properties of the substrate. It has a high rate of surface diffusion and crystallization easily from the source of vapor even at low substrate temperature.

3.1.1. X-ray diffraction (XRD):

At first, XRD studies were carried out in order to get an idea of the nature of the crystal structure of **Ag, Sn, and Zn** metals prepared by an atmospheric plasma jet system.

Depending on the sample's phases, the X-ray beam diffracts at distinct angles with respect to the incident beam. Figure 3a, b, c, and d depict the X-ray pattern for Ag, Sn, and Zn metals, respectively. Diffraction peaks broaden when the crystal size decreases to the nanoscale range, and the width of the peak is directly correlated with the size of the nanocrystalline according to the Debye Scherrer formula.

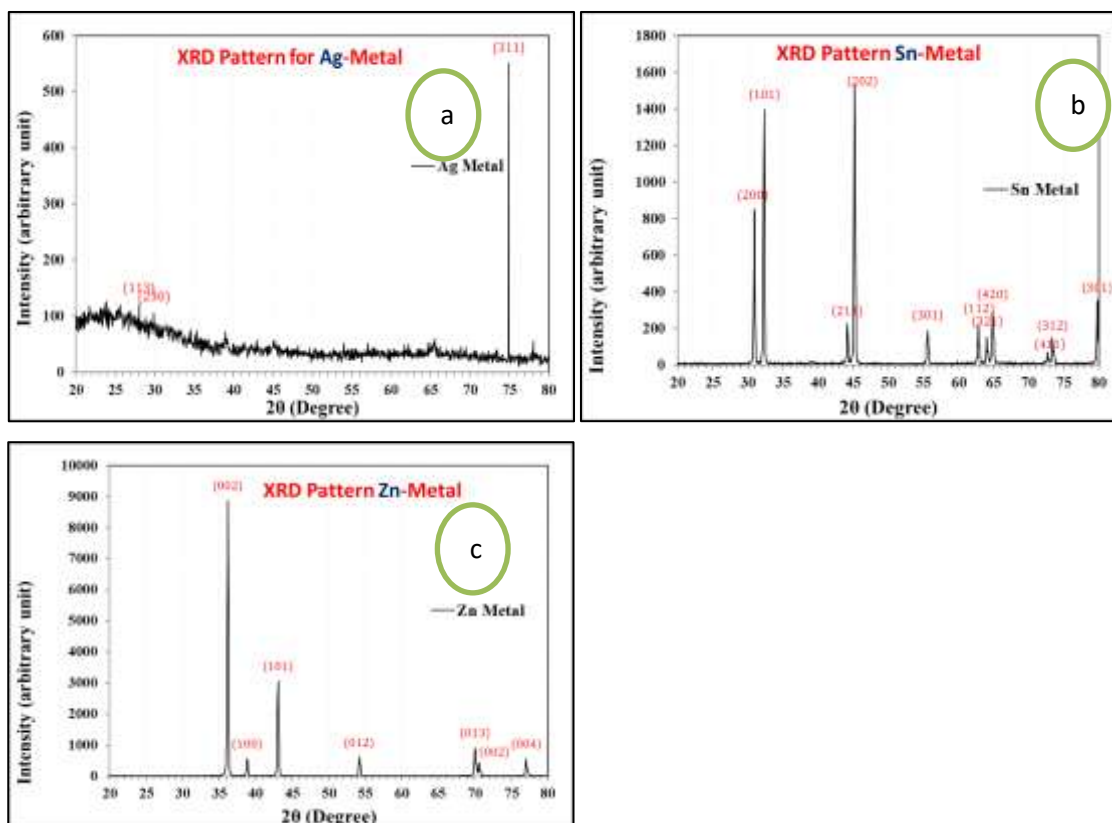


Fig. 3. XRD spectra of a) Ag, b) Sn and c) Zn metals.

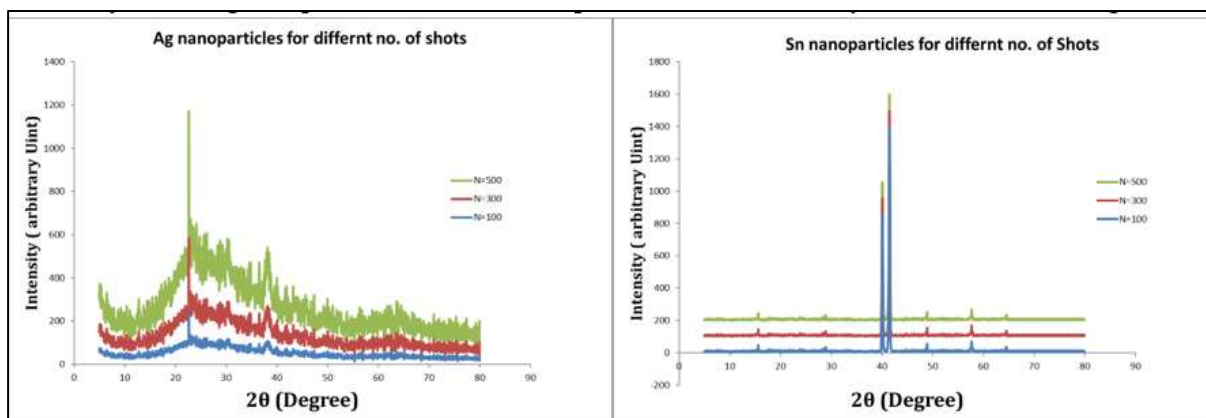
It is observed from the figure 3, That Ag, Sn and Zn metals deposited are the polycrystalline structure.

The flowing table explains the peaks for Ag, Sn and Zn metals:

Table 2: XRD patterns for Ag, Sn and Zn metals compared with standard reference data [24].

material	2 θ (Deg.)	FWHM (Deg.)	dhkl Exp.(Å)	dhkl Std.(Å)	hkl
Ag metal	28.0881	0.1712	3.1743	3.1698	113
	29.9479	0.1511	2.9813	2.9877	230
	75.4085	0.1000	1.2595	1.2655	311
Sn Metal	30.9394	0.1683	2.8880	2.8881	200
	32.3309	0.1904	2.7668	2.7662	101
	44.1896	0.1640	2.0479	2.0480	211
	45.2060	0.1829	2.0042	2.0041	202
	55.6230	0.2139	1.6510	1.6511	301
	62.8631	0.1712	1.4771	1.4771	112
	64.8834	0.1995	1.43595	1.43594	321
	72.7046	0.1483	1.29954	1.2995	420
	73.444	0.1745	1.28827	1.2882	411
Zn Metal	79.8228	0.1519	1.20059	1.2005	312
	38.8678	0.1943	2.3152	2.3151	100
	43.0893	0.1931	2.0976	2.0975	101
	54.2197	0.1992	1.6904	1.6903	012
	70.0429	0.2132	1.3423	1.3421	013
	70.5710	0.1786	1.3335	1.3334	002
	77.061	0.1835	1.23657	1.2365	004

When the Ag, Sn and Zn metals are prepared as nanoparticles at different times using a plasma jet system using a drop cast method with a temperature of 60 °C an X-ray Pattern is shown in Figure 4.



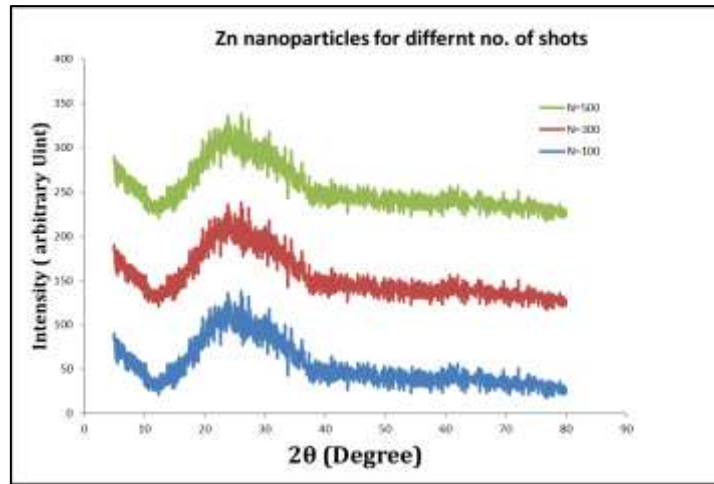


Fig. 4. XRD spectra of a) Ag, b) Sn, and c) Zn nanoparticles prepared by drop casting

The following table explains the peaks for Ag, Sn and Zn nanoparticles.

Table 3: XRD for Ag, and Sn nanoparticles comparing with Standard[104].

material	2θ (Deg.)	FWHM (Deg.)	dhkl Exp.(Å)	dhkl Std.(Å)	hkl
Ag nanoparticle	22.0881	0.1712	3.1743	3.1698	111
	38.2479	0.1511	2.9813	2.9877	230
Sn nanoparticle	30.9394	0.1683	2.8880	2.8881	200
	32.3309	0.1904	2.7668	2.7662	101

Diffraction peaks are well compatible with those of the given data COD (open crystallography database) [30]. We also note that the diffraction peaks and their positions shift toward other nearby diffraction angles, and the reason for this is due to an improvement in the crystalline with the acquisition of deposited atoms to additional energy resulting from thermal treatment and re- diffusion in low energy sites and having new sites.

The effect of drop cast plays an important role in the determination of metals films structure fabricated on the glass substrate[33].

3.1.2. Atomic force microscope (AFM):

The morphological characteristics of the Ag, Sn, and Zn nanoparticles using PLD technology with different pulse numbers (100, 300, and 500), have been studied by Atomic force microscope (AFM) to monitor the nanostructure under the influence of the different value of X to study the effect of preparation time on the size of nanoparticles and the topography of the surface as shown in Figures 5-7, which shows the three-dimensional image of the surface morphology of the metals nanoparticles.

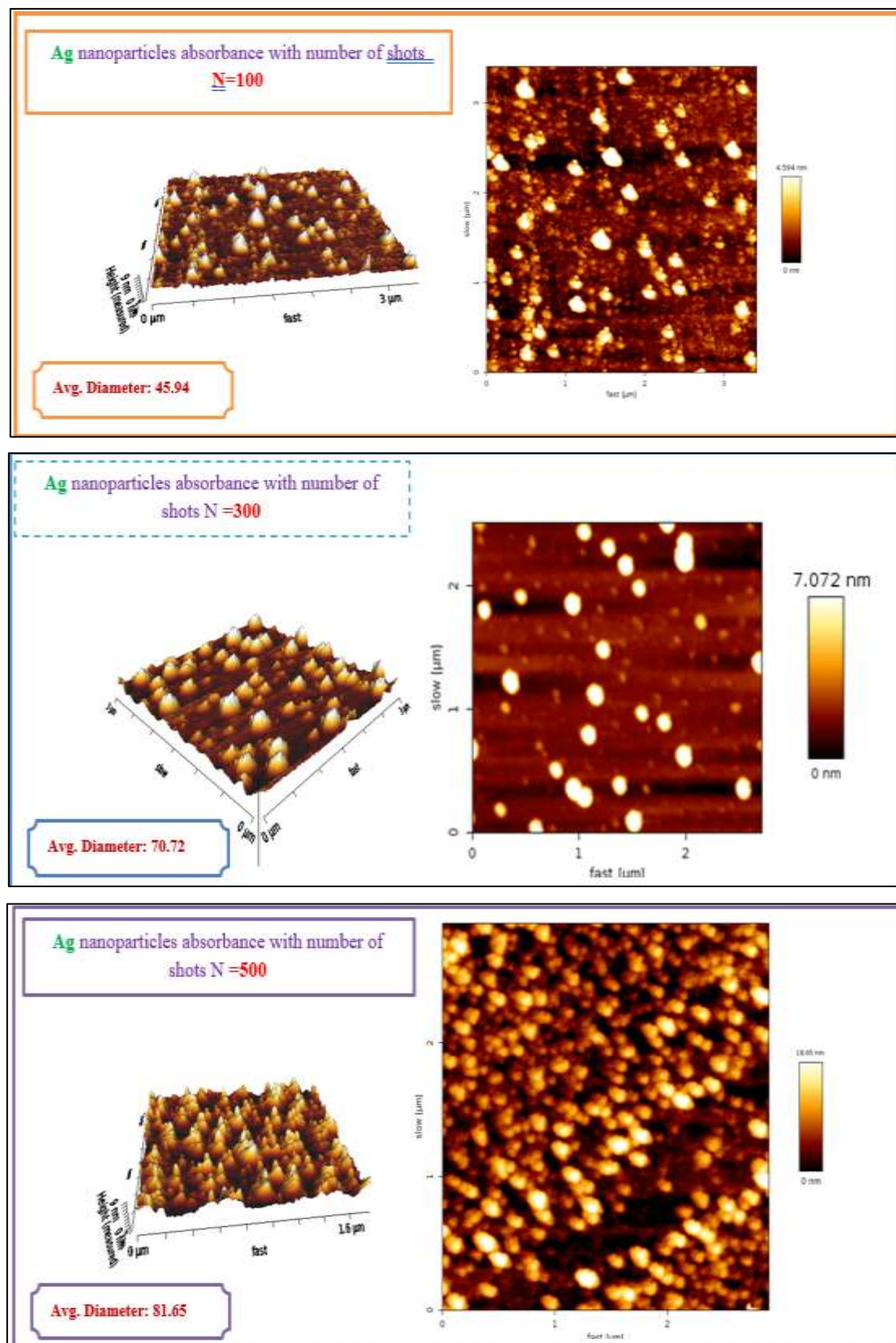


Fig. 5. 3D AFM images of Ag nanoparticles prepared with PLD deposited by drop casting at 60 °C different pulse numbers 100, 300 and 500 pulses.

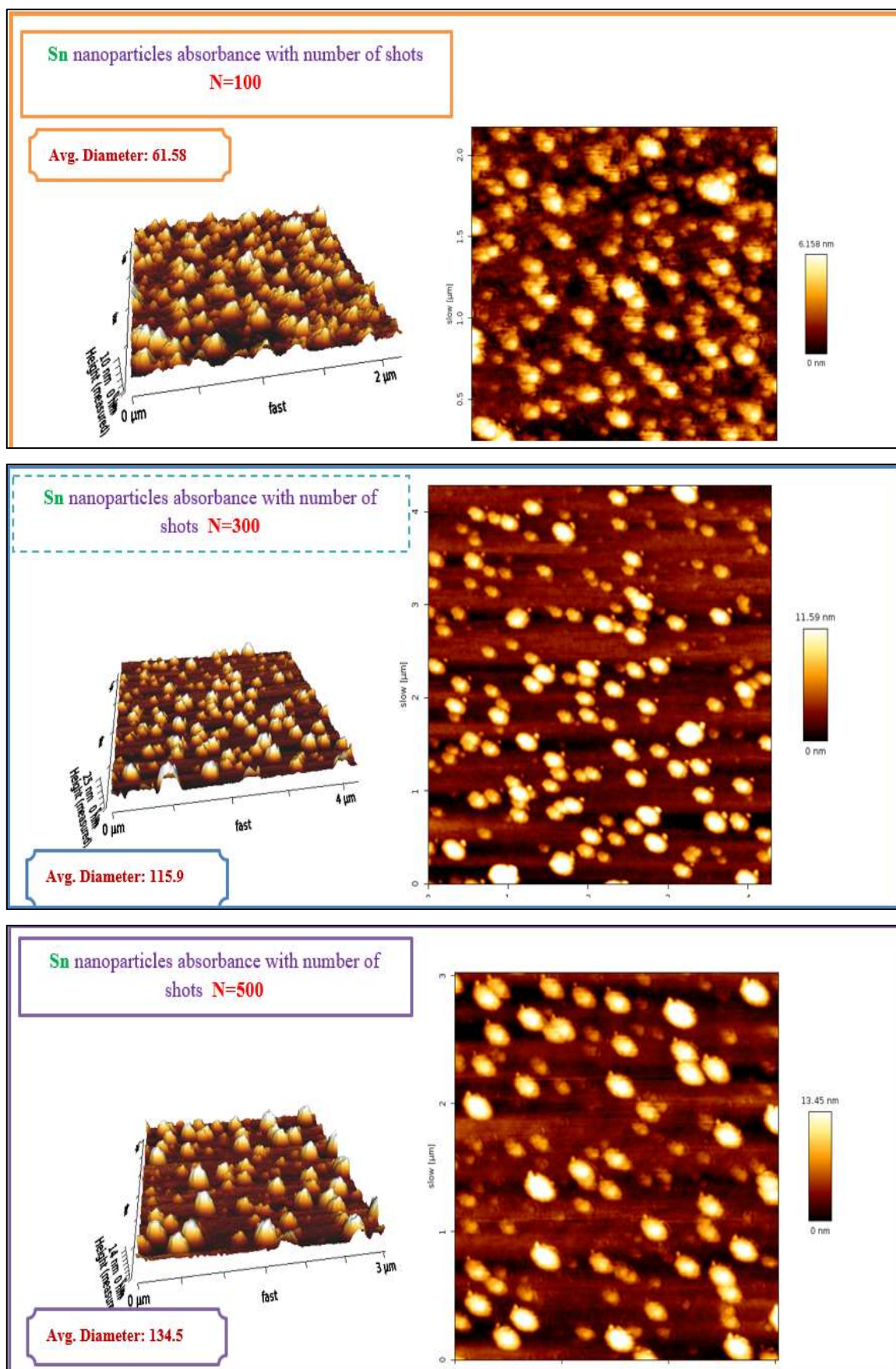


Fig. 6. 3D AFM images of Sn nanoparticles prepared with PLD using drop casting at 60 oC with no.of shots 100 , 300 and 500 pulses.

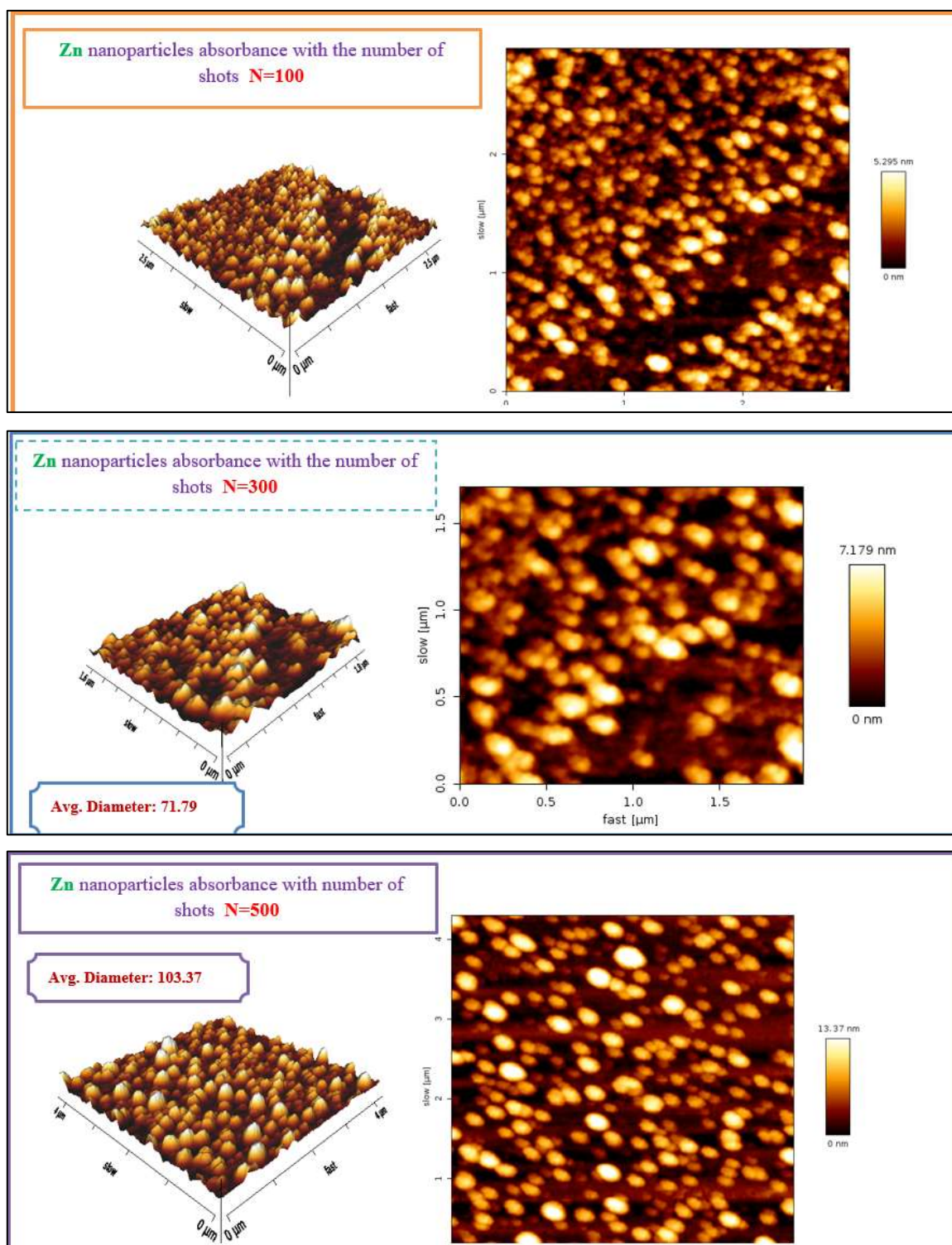


Fig.7. 3D AFM images of Zn nanoparticles prepared with PLD and deposited by drop casting at 60 °C with no. of shots 100, 300 and 500 pulses.

The influence of time preparing nanoparticles on thin-film of metals has been investigated on the topography of the thin film's surface and the average nanoparticles size at the different time preparation, respectively.

3.2 Optical properties:

The optical properties of Ag, Sn, and Zn nanoparticles solution grew by distilled water involve the Absorption, optical energy gap (E_g), refractive index, and the real and imaginary conductivity. Study the effect of the variation of time (10,15,20, and 25 min) prepared by the APPJ technique.

3.2.1 The Absorption spectrum:

The optical properties of the nanoparticles prepared by PLD have been measured by UV-VIS Spectrophotometer. Figure 8 show the Absorption versus wavelength for those nanoparticle solutions deposited. The Absorption of solution deposited in the UV-Visible region (300-1100nm) using distilled water has been examined, and it found that all solution prepared by the PLD method has high Absorption at 300 nm. The visible average Absorption for those solutions increases with varying various factors that basically is a result of the decrease in the concentration of nanoparticles in which preparing as a solution using the applied voltage of 1360 V and Argon gas with flow rate of 4 L/min at different time. Optical Absorption depends on the crystal structure of the nanoparticle solution prepared and on the nature of the topography of the surface.

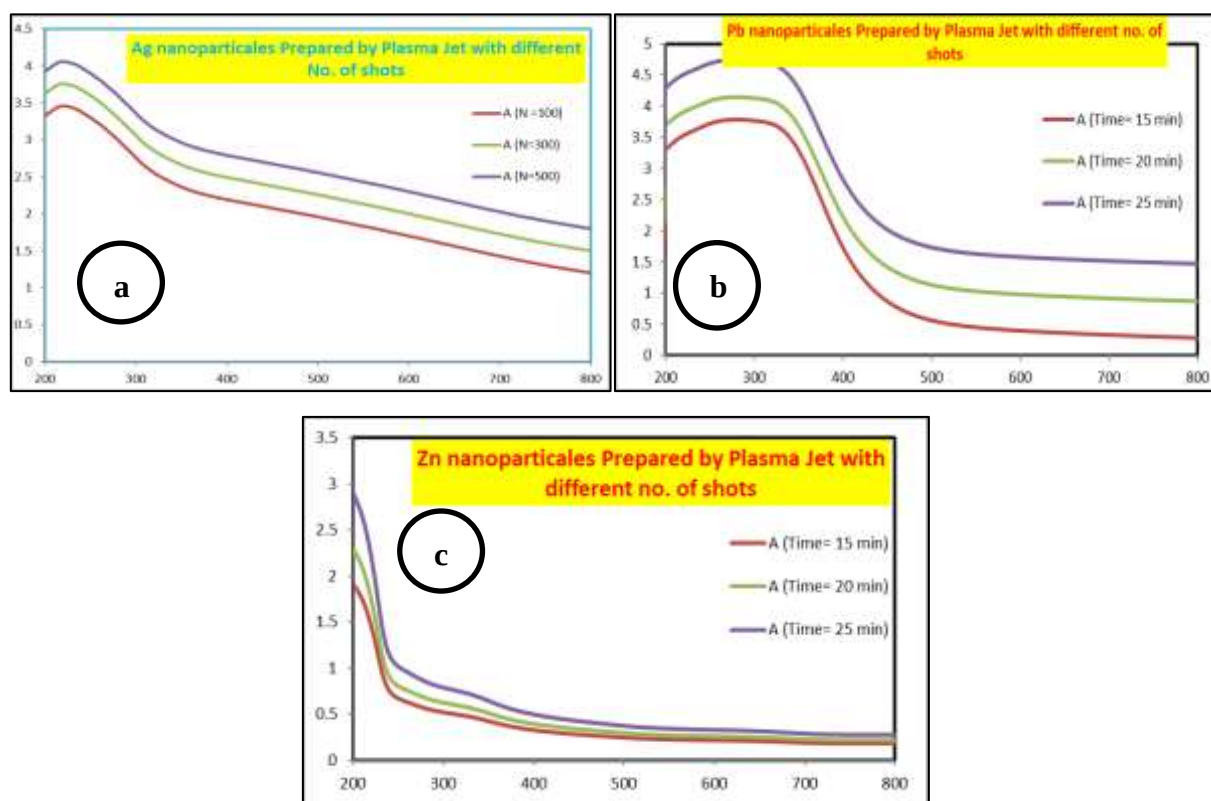


Fig. 8. Absorption spectrum as a function of wavelength for a) Ag-nanoparticle, b) Pb-nanoparticle and c) Zn-nanoparticle, prepared at different times using the PLD technique.

It is clear from the figure that at a certain wavelength, the Absorption increases with increasing the concentration (No. of particles). It is clear that when the time of preparation increased, the number of particles targeted increased, which in turn led to an increase of particles ejected toward the substrate. These ejected particles have high kinetic energy when

it absorbs the high energy of the plasma jet, and the concentration of the solution will increase, and thus the Absorption will increase with increasing concentration. This behavior is compatible with the behavior of optical Absorption relationship with the energy of pulsed laser deposition and we noted that as the laser pulse energy increases, the film typically becomes thicker and absorbs more light, and the optical band gap may also increase, as seen in published studies. In other words, higher pulse energy often leads to a direct increase in absorption and thickness, and the energy gap may consequently increase in some deposited films [34].

3.2.2 The Optical energy gap (Eg):

To understand the optical and electrical properties of nanoparticle solutions made using the PLD technique, it is essential to examine and measure the average value of the energy gap. The distribution of atoms in the crystal lattice and the composition and arrangement of nanoparticles both affect this value. For instance, the graph of $(h\nu)^{1/n}$ vs. h has been shown for different preparation conditions in figure 9a,b,c, and d, where the optical band gap value indicates the point of intersection between the straight line and the axis of photon energy.

The bandgap of the material and the exponent n depend on the kind of optical transition; for crystalline semiconductors, n can be $1/2$, $3/2$, 2 , or 3 depending on whether the transfer transitions are directly permitted, direct prohibited, indirectly allowed, or indirectly forbidden. The crystal structure is affected by the circumstances and type of the thin film structure, as well as the technique of preparation.

The plot of $(\alpha h\nu)^2$ versus $(h\nu)$ for Ag, Pb, and Zn nanoparticles solutions with different X is shown in figure 8.

The Tauc relation [34] may be used to determine the direct energy gap for samples:-

$$\alpha h\nu = B(h\nu - E_g)^{1/2} \quad (1)$$

Where B is a constant that varies depending on the material, h is Planck's constant, and ν is the frequency of photons.

Using the connection between $(\alpha h\nu)^2$ and photon energy $(h\nu)$, we calculate the energy gap value by extending the straight portion of the curve to cut the photon energy axis at the point $(\alpha h\nu)^2 = 0$, with a thickness measured using Fizeau Fringes equal to 100 nm.

The optical energy gap for direct transmission of Ag, Pb, and Zn nanoparticles with varying numbers of shots is displayed in Figure 9.

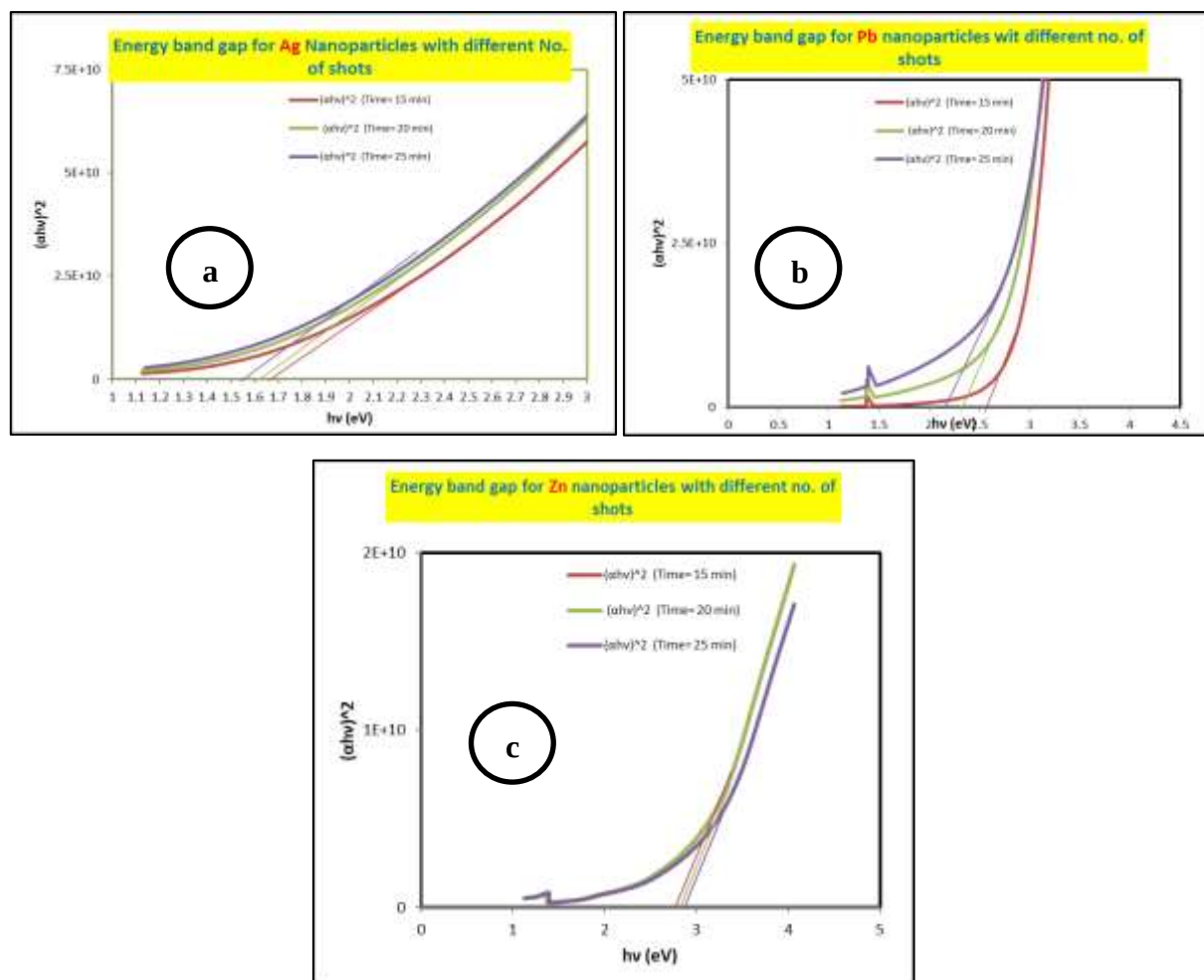


Fig. 9. Variation of $(\alpha h\nu)^2$ with the photon energy for a) Ag-nanoparticle, b) Pb-nanoparticle, and c) Zn-nanoparticle, prepared at different times using the PLD technique.

The following tables show the variation of time preparations with energy gap in PLD techniques for Ag, Pb and Zn nanoparticles solutions as: -

Table 4. Energy gap values for Ag-nanoparticles.

Element = Ag Nanoparticles	Under vacuum			E(Standard)
No. of Shots	100	300	500	1.6
Energy gap(eV)	1.59	1.62	1.69	

Table 5. Energy gap values for Pb-nanoparticles.

Element = Pb Nanoparticles	Under vacuum			E(Standard)
No. of Shots	100	300	500	2.8
Energy gap(eV)	2.4	2.6	2.75	

Table 6. Energy gap values for Zn-nanoparticles.

Element = Zn Nanoparticles	Under vacuum			E(Standard)
No. of Shots	100	300	500	2.6
Energy gap(eV)	2.75	2.81	2.86	

According to the aforementioned tables, the energy gap value at the experimental value of the E_g range is almost within the conventional E_g range. As the time of preparation increases, the concentration of nanoparticles in solution increases. This led the absorbance of the solution to increase, so the energy gap increased.

3.3. Gas sensor Performance of Ag-, Sn-, and Zn-/TNTs:

The response times for Ag-, Sn-, and Zn-/doped TNTs, which were prepared at room temperature, Additionally, Ag-, Sn-, and Zn-/TNTs, which were prepared as gas sensors, showed response times for H_2S sensing of less than 50 seconds at room temperature. Even though the recovery times shown in Figure 10.

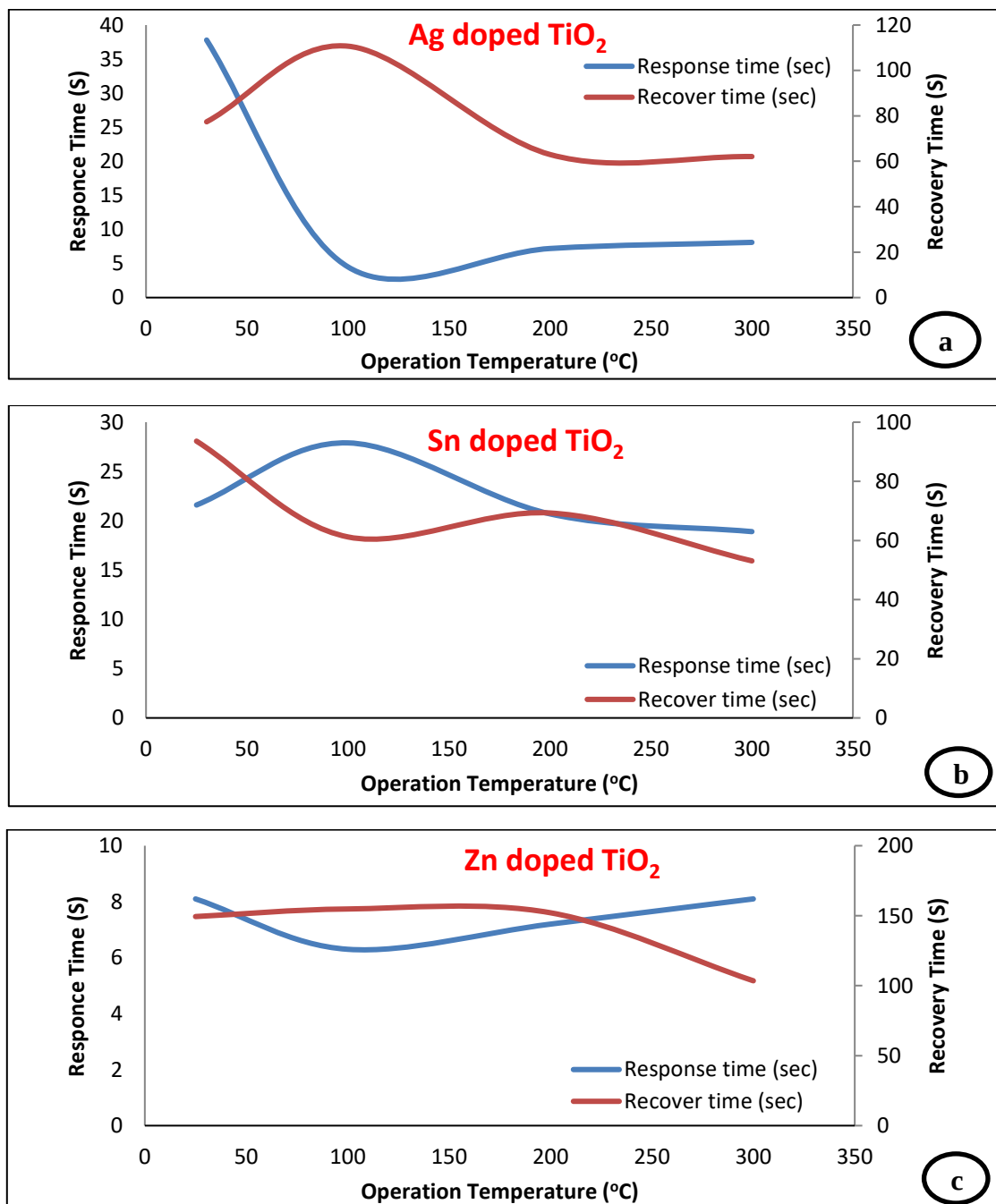


Fig. 10. Response and recovery times of TNTs prepared with different dopant metals : (a) Ag , (b) Sn, and (c) Zn

Figure 11 shows the gas sensitivity of the Ag/TNTs sensor to H_2S at different operating temperatures. The sensitivity of Ag/TNTs sensors about 26%, while The sensitivity of Sn/TNTs sensors about 11% and The sensitivity of Zn/TNTs sensors about 10% the highest sensitivity at room temperature that was prepared at N=500 shots .The enhanced sensitivity of Ag-doped TNTs may be attributed to improved charge transfer and surface plasmon effects

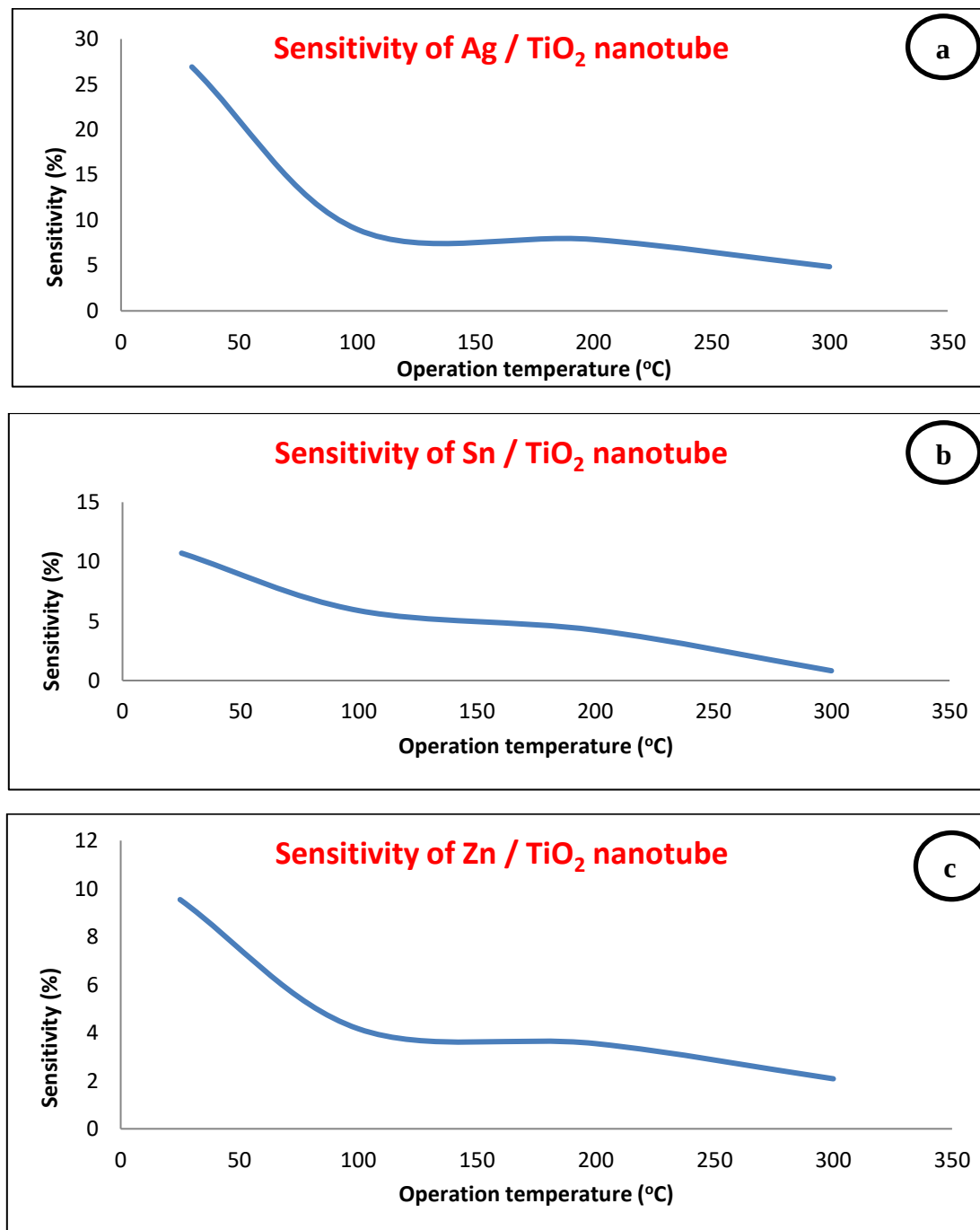


Fig. 11. Sensitivity of of TNTs prepared at different doping metals : (a) Ag , (b) Sn, and (c) Zn

Conclusion

In this study, Ag-, Sn-, and Zn-doped titanium dioxide nanotubes were successfully fabricated by pulsed laser deposition, demonstrating that this method can effectively modify the structural and functional properties of TiO₂ nanotube systems. The incorporation of different dopants significantly improves the nanotubes' surface characteristics, crystallinity, and optical response, which makes them promising for applications in photocatalysis, sensing, and energy conversion. Overall, the results show that doped TiO₂ nanotubes produced by pulsed laser deposition offer a flexible platform for tailoring nanomaterial performance for advanced technological uses.

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Enhanced Optical Properties and Photoluminescence of $\text{Eu}_2\text{O}_3/\text{PMMA}$ Nanocomposite Films

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Abstract:

The optical and photoluminescence properties of Eu_2O_3 nanoparticles and $\text{Eu}_2\text{O}_3/\text{PMMA}$ nanocomposite films under UV irradiation have been investigated. The absorption spectra of Eu_2O_3 nanoparticles between 200 and 1200 nm exhibit strong absorption characteristics in the UV region with a sharp edge at around 390 nm, suggesting a band gap value of 3.38 eV from the Urbach tail related to defect and surface states. Under 337 nm excitation, Eu_2O_3 nanoparticles exhibited strong blue emission at 385-505 nm from the defect states, while the characteristic emission lines of Eu^{3+} ions at 548 and 612 nm were weak. Films of Eu_2O_3 nanoparticles dispersed in a PMMA matrix exhibited UV-blue emissions from the defect and the PMMA matrix under excitation with a 305 nm UV LED, green emissions from the $^5\text{D}_1$ to $^5\text{D}_0$ transition of Eu^{3+} ions, and a strong red emission at around 631 nm from the $^5\text{D}_0$ to $^7\text{F}_2$ transition of Eu^{3+} ions. The chromaticity coordinates of the photoluminescence spectra of the $\text{Eu}_2\text{O}_3/\text{PMMA}$ films under excitation with the 305 nm LED were found to be (0.25, 0.425), indicating the cyan emission of the films. Thus, $\text{Eu}_2\text{O}_3/\text{PMMA}$ films are identified as a potential phosphor material for cyan LED emission when excited by UV LED sources,

Keywords: Eu_2O_3 nanoparticles , PMMA, Nanocomposite films , Photoluminescence , Optical properties , UV-Visible spectroscopy, UV-LED excitation , Rare-earth oxides , Europium ions (Eu^{3+}) .



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1. Introduction

Due to their flexibility, lightness, and processability, polymer–inorganic nanocomposites have been of intense interest in optoelectronics. By embedding inorganic nanoparticles within a transparent conducting polymer matrix, it is possible to create materials that exhibit desired optical properties within the UV–visible range, which is essential for the function of a variety of optoelectronic devices [1–3]. PMMA is one of the most commonly utilized optical polymers due to its high degree of transparency from the near-UV to the near-IR region of the electromagnetic spectrum.

Rare-earth metal oxides have long been of interest in optoelectronics due to their ability to emit light at sharp emission peaks with high color purity [4–6]. The red-emitting properties of the europium-based rare-earth oxide, Eu_2O_3 , have drawn particular interest in the optoelectronic field. Eu_2O_3 has been reported to exhibit intense red emission peaks at 613–614 nm from the Eu^{3+} ion's $^5\text{D}_0 \rightarrow ^7\text{F}_2$ electronic transition under UV excitation at 240–280 nm. Additionally, studies on the incorporation of rare-earth metal ions into PMMA have revealed that the resulting materials exhibit enhanced photoluminescence properties and act as bright red-emitting films and fibers [6].

Previous studies on metal oxide–PMMA nanocomposites have demonstrated that rare-earth ions such as Eu^{3+} exhibit high photoluminescence yields and optical transparency in various polymer matrices [7]. Eu_2O_3 nanoparticles embedded within an organic resin matrix have been shown to exhibit strong red luminescence under UV light excitation [8,9]. Despite these studies on rare-earth metal oxide–PMMA nanocomposites, there remains a need to prepare and study nanocomposite films of PMMA and Eu_2O_3 nanoparticles that exhibit enhanced luminescent properties under UV-LED excitation [10,11]. Specifically, it is of interest to investigate the effect that the content and dispersion of Eu_2O_3 nanoparticles have upon the optical properties of PMMA-based films, such as UV–visible absorption, refractive index, and photoluminescence spectra. Additionally, evaluating the photostability and transparency of these nanocomposite films under continuous UV-LED excitation will provide insight into their potential as phosphor layers for UV-LED devices [3].

In this work, Eu_2O_3 /PMMA nanocomposite films were prepared using the method described in the following section, and their structural and optical properties were investigated. The UV-Visible spectra of the nanocomposite films were used to determine their optical band gap and dispersion parameters, and their photoluminescence spectra were recorded under UV-LED excitation to investigate their photoluminescence emission spectra and color characteristics as a function of the amount of Eu_2O_3 nanoparticles within the PMMA matrix. Results from this study will help reveal the role that Eu_2O_3 nanoparticles play in PMMA-based films in enhancing their photoluminescence properties and indicate their potential use as red-emitting phosphor layers for various UV-LED devices.

2. Experimental Work

2.1. Materials:

PMMA granules of analytical grade and $\geq 99\%$ purity were purchased from Sigma-Aldrich. Europium (III) oxide (Eu_2O_3) nanopowder, $\geq 99.9\%$ trace metals basis, was purchased from Sigma-Aldrich and used without further purification. The high purity of these components was used to ensure that no extraneous impurities could affect the properties of the prepared nanocomposites.

2.2. Preparation of Eu_2O_3 /PMMA Nanocomposite:

Polymethyl methacrylate (PMMA) is the most commonly used polymer in the methacrylate family. The chemical structure of PMMA is shown in Figure (1) [12].

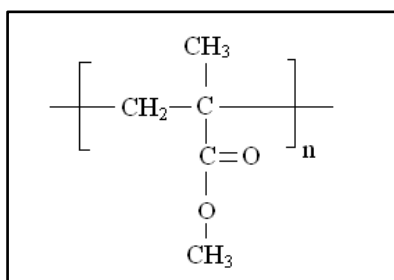


Figure (1): Structure of Polymethyl methacrylate (PMMA)

PMMA (1.00 g) was dissolved in 10.0 mL chloroform (HPLC grade) by stirring at 50°C (~ 250 - 300 rpm) until fully dissolved (~ 30 - 60 min) to afford a 10 wt% (w/v) solution. Eu_2O_3 nanoparticles (10.0 mg) were pre-dispersed in ~ 1 mL of chloroform and added to the PMMA solution. The resulting mixture was probe-sonicated and stirred at 50°C for 10 min. After degassing of the mixture under reduced pressure ($\sim 10^{-1}$ mbar) for 10-15 min, the mixture was coated on glass substrates. The PMMA and PMMA: Eu_2O_3 (1 wt%) films were evaporated in a dry oven at 50°C for 24 h and further dried under vacuum ($\sim 10^{-2}$ - 10^{-3} mbar) for 2-4 h. The spectra of pristine PMMA and PMMA: Eu_2O_3 (1 wt%) thin films were recorded at room temperature using UV-Vis spectrophotometry (200-800 nm).

3. Results and Discussions

3.1. Optical Properties:

The absorbance of the samples was measured using an OPTIMA SP-3000 UV-VIS spectrophotometer supplied by Optima Company, covering a range from 200 to 1200 nm. The absorption spectrum of Eu_2O_3 was measured after dissolving it in HCl with a concentration of 0.01 g in 10 mL.

The absorption spectrum of the nanoparticles of Eu_2O_3 is shown in Figure (2), which shows low absorptance of Eu_2O_3 NPs in the visible and near infrared regions; however, the absorptance in the ultraviolet region is high. Also, the absorption spectrum shows that the fundamental absorption edge (the boundary between the high-absorption region and the low-absorption window) is sharp in the visible, at a wavelength of about 390nm.

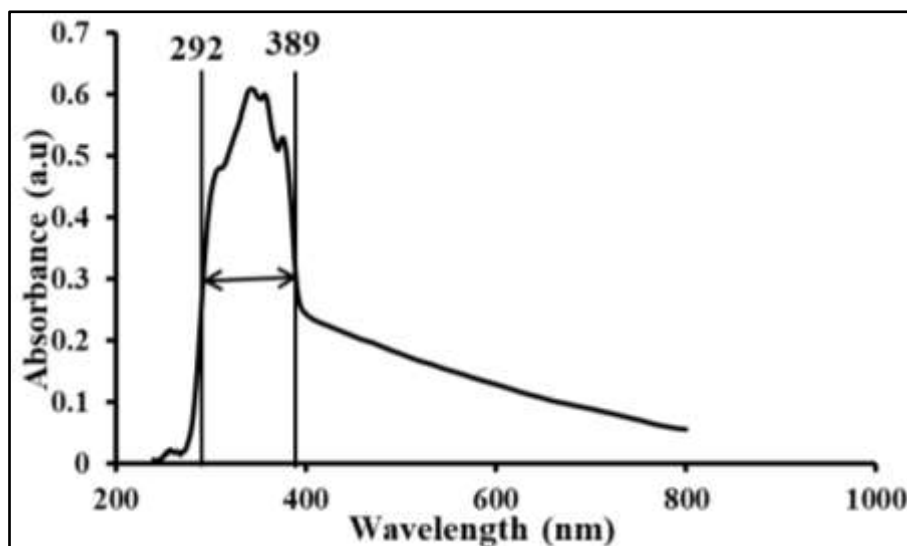


Figure (2): Absorption spectrum of Eu₂O₃ NPs

The high-absorption region (292–389 nm) is attributed to electronic transitions between the valence and conduction bands. The linear decrease observed beyond 390 nm corresponds to the Urbach tail, which arises from localized states within the band gap caused by surface effects in nanoparticles, as well as from 4f–4f transitions within the Eu ion.

The band gap was calculated using Tauc's relation and found to be **3.38 eV**, as shown in Figure (3), which is lower than the ideal values reported for Eu₂O₃ [13]. This reduction is attributed to localized energy states within the band gap arising from structural defects and surface effects in the nanoparticles, in addition to the influence of the Urbach tail [14], which extends absorption toward lower energies.

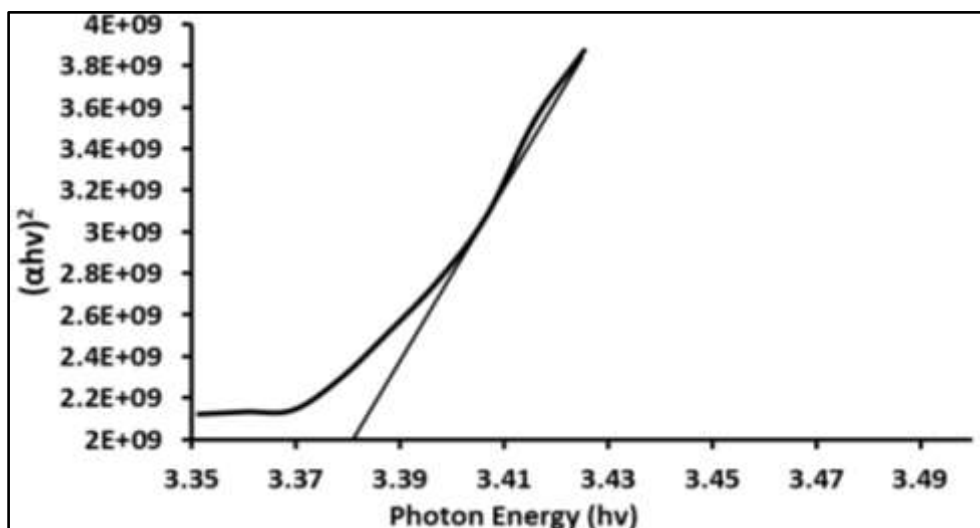


Figure (3): $(\alpha h\nu)^2$ versus Photon energy for Eu₂O₃ NPs

3.2. Photoluminescence spectrum (PL):

The Photoluminescence spectrum of the samples was measured using SL 174 SPECTROFLUOROMETER supplied by ELICO Company, covering a range from (200-900) nm, as shown in Figure (4).

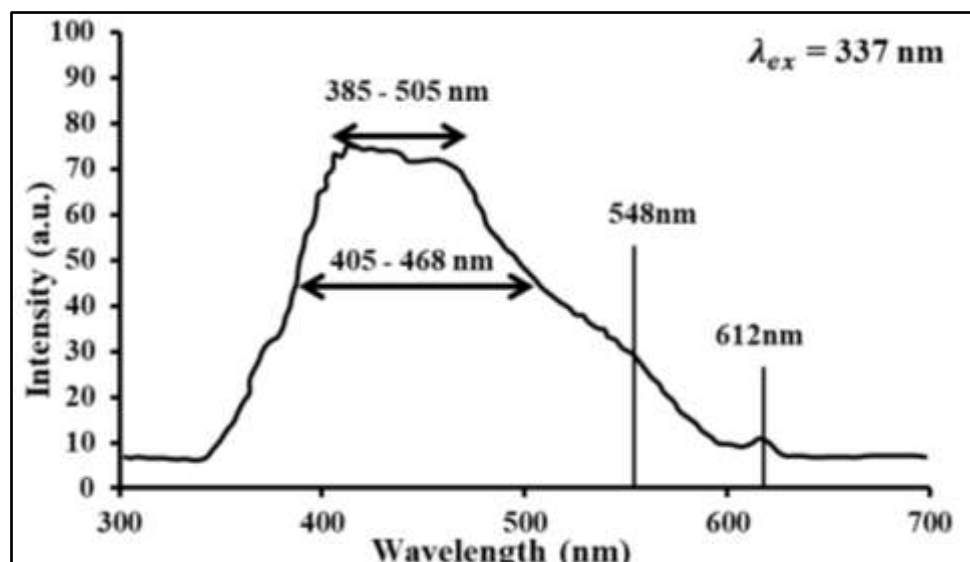


Figure (4): PL spectrum of the Eu₂O₃ NPs

Photoluminescence (PL) spectra provide information on the energy states available between the valence and conduction bands responsible for radiative recombination. The photoluminescence spectrum of the Eu₂O₃ nanocrystals solution (Eu₂O₃ nanocrystals in HCl with a concentration of 0.001 g/ml), excited by the 337nm line, is shown in Figure (4) (Irradiation was carried out at a wavelength of 337 nm, based on the maximum absorption recorded in the absorption spectrum).

The emission spectrum shows a broad band extending from 385 to 505 nm, which is attributed to localized energy states arising from defects and surface effects in the nanoparticles. Electrons are trapped in these localized states within the band gap and then return to lower-energy levels through radiative recombination, leading to the emission of lower-energy photons.

A nearly flat plateau is observed within 405–468 nm due to the overlap of several closely spaced transitions or a broad distribution of energy levels. This results from the irregular distribution of defects and variations in the local environment of Eu³⁺ ions within the lattice, causing the peak to appear broad rather than sharp [15] [16].

Additional peaks are observed at 548 nm and 612 nm [17], corresponding to internal transitions of the Eu³⁺ ion. The peak at 612 nm represents the characteristic (⁵D₀→⁷F₂) transition of Eu³⁺, but it appears weak due to quenching effects and the dominance of defect-related emission.

3.3. UV-LED illumination:

From Figure (2), the spectrum shows that the high absorption range lies between 292 and 389 nm, indicating an efficient absorption of the employed ultraviolet source, whose emission spectrum is shown in Figure (5).

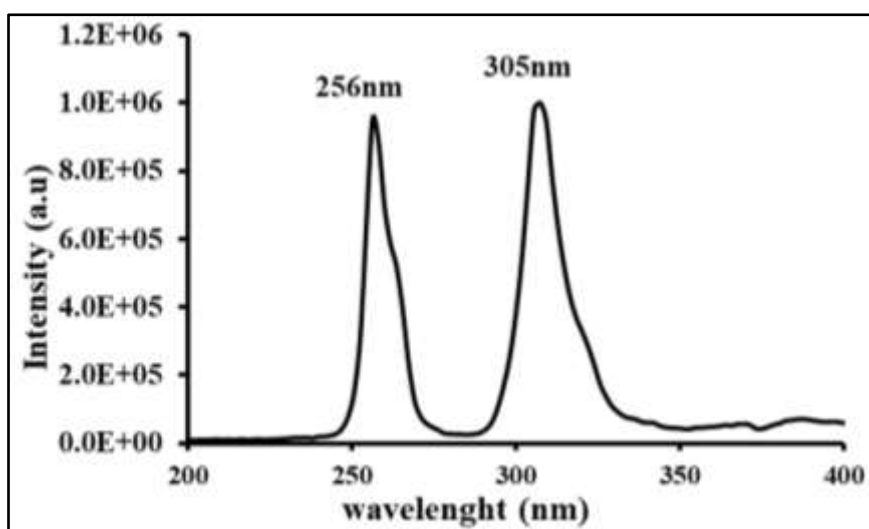


Figure (5): Emission spectrum of the ultraviolet source

The Eu_2O_3 /PMMA nanocomposite films were illuminated using a GaN UV-LED with a power of 5 mW and a wavelength of 305 nm, supplied by Fernel Company, in order to generate light from these nanoparticles. The emission spectrum of the resulting light can be observed in Figure (6).

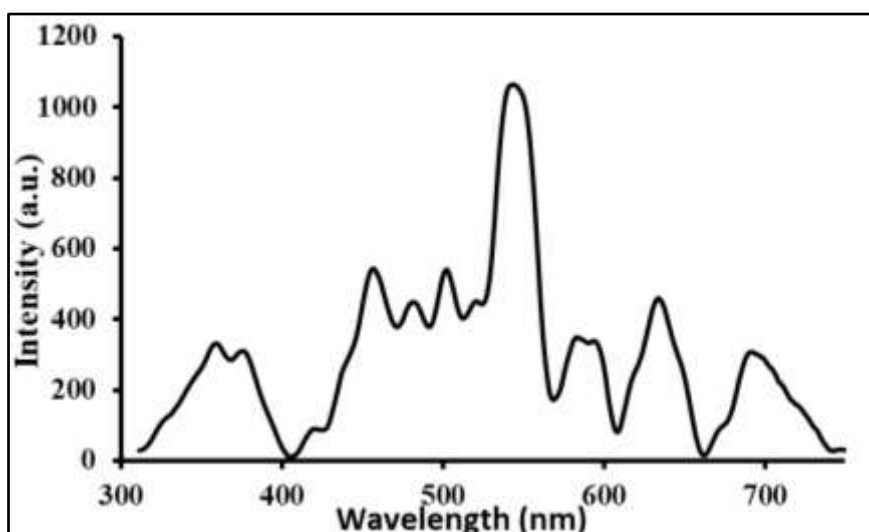


Figure (6): PL spectrum of Eu_2O_3 /PMMA NCs films

The main peaks in the spectrum are identified in Table 1.

Table (1): Main Emission Peaks of the spectrum

Peak no.	Wavelength (nm)	States
1	354	surface states and defect states
2	369	surface states and defect states
3	414	surface states for Eu_2O_3 and states for PMMA
4	436	surface states for Eu_2O_3 and states for PMMA
5	453	surface states for Eu_2O_3 and states for PMMA
6	476	surface states for Eu_2O_3 and states for PMMA

7	500	surface states for Eu ₂ O ₃ and states for PMMA
8	515	$^5D_1 \rightarrow ^7F_j$ and $^5D_0 \rightarrow ^7F_1$
9	538 (533-557)	$^5D_1 \rightarrow ^7F_j$ and $^5D_0 \rightarrow ^7F_1$
10	578	$^5D_1 \rightarrow ^7F_j$ and $^5D_0 \rightarrow ^7F_1$
11	588	$^5D_1 \rightarrow ^7F_j$ and $^5D_0 \rightarrow ^7F_1$
12	631 (617-650)	$^5D_0 \rightarrow ^7F_2$
13	688 (681-713)	$^5D_0 \rightarrow ^7F_3/ ^7F_4$

Upon excitation at 305 nm, the emission spectrum of the Eu₂O₃/PMMA film exhibits multiple peaks across the UV-visible region. The emissions in the range (354–500 nm) are attributed to defect-related states and the contribution of the PMMA matrix, where electrons are excited to higher energy levels and subsequently trapped in localized states within the band gap before recombining radiatively. The plateau observed within (414–476 nm) results from overlapping transitions and a broad energy distribution arising from structural defects and variations in the local environment. In the green region (515–578 nm), the emission peaks are assigned to Eu³⁺ transitions from the excited states (⁵D₁ and ⁵D₀) to lower levels, facilitated by energy transfer from the host matrix. The prominent red emission around 631 nm corresponds to the characteristic (⁵D₀→⁷F₂) transition, indicating a non-centrosymmetric environment around Eu³⁺ ions. Additionally, the emission near 688 nm is attributed to lower-energy transitions (⁵D₀→⁷F₃ or ⁷F₄) [18].

3.4. CIE 1931 color space:

The illumination of the Eu₂O₃/PMMA composite films was carried out using a GaNUV-LED with a power of 5 mW and a wavelength of 305nm, supplied by Fernel Company, to excite the light from these nanoparticles, as shown in Figure (5). The light that is emitted by PL should have an acceptable color in order to accurately display the colors of the objects that are to be illuminated. The color of the light can be described by the CIE colorimetry system, which utilizes the spectrum of the light and weighting it according to the XYZ color matching functions¹. The values of X, Y, and Z for the luminescence spectrum PL (luminescence by 305nm) can be calculated by determining the area under the curve for the peaks in the spectrum for the regions of red, green, and blue light. The chromaticity coordinates (x,y) for the light according to the CIE colorimetry system can be calculated from the values of X, Y, and Z according to the equations below (Eqs. 1 and 2) [19].

$$x = \frac{X}{X + Y + Z} \dots \dots (1)$$

$$y = \frac{Y}{X + Y + Z} \dots \dots (2)$$

Where X, Y, and Z are the areas under the curve of the emitted red, green, and blue in the luminescence spectrum, respectively. Any color of light can be expressed in terms of its chromaticity coordinates (x, y) on the CIE 1931 (x, y) diagram of chromaticities. The light

emitted from Eu₂O₃/PMMA has tristimulus coordinates of (x, y) = (0.25, 0.425). This color is represented by a marked point on the CIE 1931 (x, y) diagram as a shade of blue- green (cyan) within the perceivable colors. The color temperature of the white light emitted by the Eu₂O₃/PMMA can be determined using McCamy's approximation algorithm for the correlated color temperature (CCT) from the chromaticities (x, y) [20].

$$CCT = -449n^3 + 3525n^2 - 6823n + 5520.33 \dots (3), CCT \text{ in (kelvin)}$$

Where **n** is equal to:

$$n = \frac{(x - 0.332)}{(y - 0.185)} \dots (4)$$

Using the determined values of the variables x and y, the color temperature of the produced light was found to be about 1931 K. The measured chromaticity coordinates of the light produced by the emission lines in the PL spectrums plotted on the chromaticity diagram of Figure (4) indicate that the light is within the green-blue region of the spectrum, which confirms the PL measurements of the color of the light being emitted from the Eu₂O₃/PMMA exposed to the 320 nm lamp source, as seen in the photograph of Figure (4).

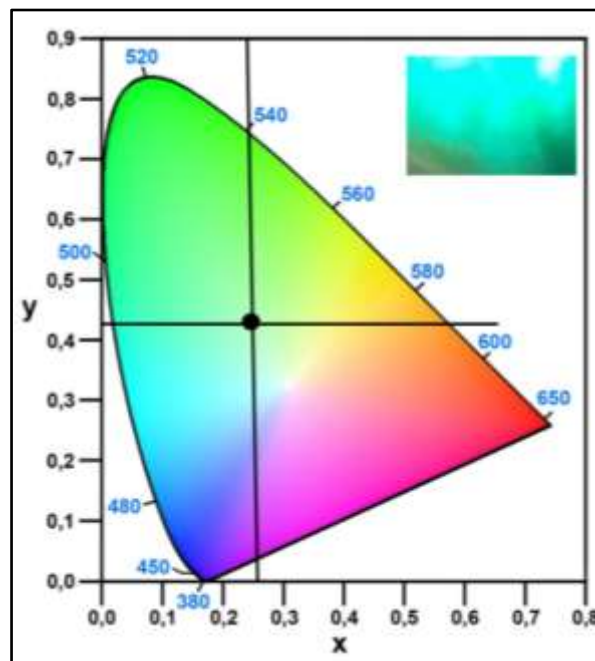


Figure (7): The CIE Chromaticity Diagram with the chromaticity coordinates of the emitted light

Conclusions

The optical and photoluminescence (PL) characteristics of Eu₂O₃ particles confirm its effectiveness as a UV-absorbing and color-converting component within a PMMA matrix. The UV-Vis absorption spectrum of Eu₂O₃ exhibits a strong absorption edge at around 390 nm, suggesting a band gap of 3.38 eV, which is lower than the band gap of bulk Eu₂O₃ due to defect states within the Eu₂O₃ crystals. The PL spectrum of Eu₂O₃ particles exhibited a broad PL band at around 400 nm due to these defect states, with very weak Eu³⁺ characteristic emission lines. When dispersed within PMMA and exposed to 305 nm UV-LEDs, the

nanocomposites exhibited PL emissions in the UV, blue, green, and red spectrums. The red emission originated from the $^5D_0 \rightarrow ^7F_2$ electronic transition of Eu^{3+} ions. The CIE chromaticity diagram coordinates of the as-synthesized nanocomposite films are approximately (0.25, 0.425), with a color temperature of 1931 K, indicating that the nanocomposite materials emit a cyan color. These characteristics indicate the potential of these nanocomposite materials to act as phosphor materials for UV-LEDs.

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Evaluation of radioactive contamination in Soil samples at Al-Jadriya region/ Baghdad

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Abstract:

In this work, the evaluation of the specific activity of Ra-226 (186 keV), Bi-214 (609 keV), Cs-137 (662 keV), Ac-228 (969 keV) and K-40 (1460keV) in some samples of soil at Al-Jadriya region have been done by gamma-ray spectroscopy with NaI(Tl) scintillation detector. The research included 5 samples from within the University of Baghdad and 5 samples from different areas adjacent to the university. The range of Ra-226 specific activity was (0) Bq/kg for the samples (N3 and N4), while the highest specific activity was (77.11) Bq/kg for sample (W5). The lowest specific activity of radioactive elements was found in element Cs-137. Its highest specific activity was 17.48 Bq/kg in sample (W2), and the lowest value was 3.82 Bq/kg in sample (N2). The specific activity values of radioisotopes were different for various regions, but the average results, it was found the values of samples within the University of Baghdad were higher than the samples in the areas adjacent to the university, and all of them were within the permissible limit, except the specific activity of K-40 in the region within the university, was higher than the permissible limit. The average specific radioactivity of the samples was calculated for the following radioactive elements: Ac-228 (969 kV) 54.11 Bq/kg, Cs-137 (662 kV) 12.81 Bq/kg, Ra-226 (186 kV) 5.04 Bq/kg, K-40 (1460 kV) 381.38 Bq/kg, and Bi-214 (609 kV) 91.01 Bq/kg. All these values were within acceptable limits,

Keywords: *Gamma-Ray Spectroscopy, Al-Jadriya Baghdad, Radiation Hazard Index, Radionuclides.*



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Introduction

The presence of radioactive chemicals, whether unintentional or undesirable, on surfaces or within solid, liquid, or gaseous materials (including the human body) is known as radioactive contamination [1]. There are two types of radiation in the environment: man-made and natural. Man-made radiation results from nuclear weapons tests and nuclear accidents, while natural radiation consists of terrestrial and cosmic components [2]. The intensity of cosmic radiation, caused by cosmic rays, varies with altitude and latitude. The decay of U-238, Th-232, and K-40 is the primary source of natural terrestrial radiation in the Earth's crust. The concentration of naturally occurring radioactive elements in soil varies depending on the type of rock from which it is composed [3]. Approximately 87% of human radiation exposure comes from natural sources, while the remainder comes from human-made sources [4].

The First Gulf War (1980–1988) and the Second Gulf War (1991) subjected Iraq to intensive bombing with both conventional and prohibited weapons, including cluster bombs and bombs coated with depleted uranium alloys. As a result, Iraqi soil was contaminated with large quantities of radioactive material, which spread throughout the central and southern parts of the country, including uranium-238, a radioactive and toxic chemical [5]. If the repair is not perfect, the resulting change will spread to other cells and may eventually cause cancer. Hereditary diseases may develop if the altered cells are the ones that transmit genetic information to the offspring of the affected person [6].

The HPGe detector was used in a previous study to examine radiation hazards and levels of natural radioactivity in the topsoil of Sadr City. According to the results, average concentrations of ^{238}U , ^{232}Th , and ^{40}K were generally below international limits, although some sites recorded elevated concentrations of ^{40}K and lifetime cancer risk values close to or above the proposed international limits [7]. The study's assessment of radiation risk indicators, lifetime cancer risk, and radon-related exposures, with the exception of one sample taken near the Iraqi atomic energy facility in Tuwaitha, showed that the majority of samples were within international safety limits [8]. The study evaluated radionuclide transfer in green pepper, cucumber, celery, basil, and mint by analyzing soil, roots, stalks, and leaves, showing variations in the accumulation of U-238, Th-232, and K-40 among different plant species [9].

Trace amounts of radioactive elements are present in almost every material that surrounds us. As a result, humans are exposed to low levels of background radiation, which affects the environment and may persist for years. This can affect the genetic makeup of both humans and animals, leading to genetic defects that manifest in subsequent generations [10]. Because many naturally occurring radionuclides, as well as the fission and activation products of nuclear reactors, emit gamma radiation, gamma spectrometry is essential for tracking radioactive materials in the environment. The ability to identify and measure the activity of different radionuclides in a single counting source simultaneously is one of the advantages of gamma spectrometry [11].

This study aims to use NaI (Tl) detector technology to calculate the natural radioactivity of radium-222, bismuth-241, cesium-137, actinium-228, and potassium-40 for selected samples, and to use gamma-ray spectroscopy to identify the most important risk factors for radioactive elements that cause harm to human health. This study aims to assess the levels of radioactive contamination in soil samples taken from the Al-Jadriya neighborhood in Baghdad by measuring the concentrations of radioactivity of artificial and natural radionuclides and comparing the corresponding radioactive hazard indicators with internationally permitted limits.

2. Materials and methods

2.1. Collection and preparation of samples:

This study was conducted in the Al-Jadriya area of Baghdad Governorate. Ten soil sampling points were used at different locations within the area, as shown in Figure 1 and Tables 1 and 2. Five soil samples were taken from different locations within the campus, and five more samples were taken from adjacent areas at varying distances. Each soil sample weighed one kilogram. Samples were collected at depths of 10 to 15 cm from the surface soil. These soil samples were prepared for a number of laboratory testing. It was first crushed and then dried for eight hours at 105 degrees Celsius in the oven. After that, a 300 μ m mesh sieve was used to filter the samples. Before additional examination, 1000 grams of the resulting homogenous soil samples were carefully put into a polypropylene beaker, weighed, sealed, and kept for at least four weeks. The uranium-238 and thorium-232 isotopes had sufficient time to reach equilibrium with their radioactive products during this period.

A technique called gamma-ray scintillation spectroscopy, which Kellin described in detail, was used to measure the radioactivity. This technique is frequently used to determine the levels of potassium, thorium, and uranium in geological samples. The current study used a gamma-ray spectrometer equipped with a 3 $\times$ 3-inch NaI (Tl) detector, specifically an ORTEC-905-4 model, to measure the natural radioactivity in the area's soil. For 662 kV gamma rays emitted by cesium-137, the control percentage achieved was typically less than 7.5%.

In this study, potassium, uranium, and thorium were quantified using specific spectral peaks. Uranium was quantified using the 1.76 MeV peak of bismuth-214, thorium using the 2.62 MeV peak of thallium-208, and potassium using the 1.46 MeV peak of radioactive potassium. Each sample was counted for 3600 seconds, and background readings were calculated under the same conditions and subtracted from the gamma-ray peak.

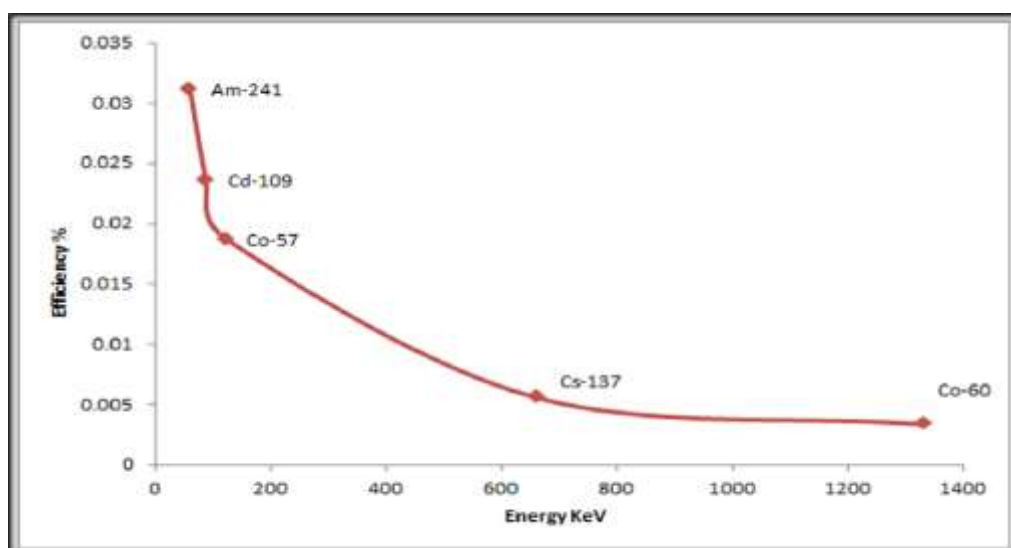


Figure (1): Efficiency calibration curve using mixed standard radionuclides in a one-liter Marinelli beaker.

Table (1): Numbers and codes of soil sample's locations within the University Baghdad.

Code	Location
W1	College of Science
W2	College of Media
W3	College of Engineering
W4	College of Agricultural Engineering Sciences
W5	College of Education for Women

Table (2): Numbers and codes of soil sample's locations next to University Baghdad.

Code	Location
N1	Al-Nahrain University Portal
N2	Central Library, Al-Nahrain University
N3	Baghdad University Arch
N4	The Ministry of Science and Technology
N5	Equestrian Club Gardens

3. Calculations

3.1 Specific Activity:

In this study, the specific activity (S) in soil samples was determined using equation (1). [12].

$$S\left(\frac{\text{Bq}}{\text{kg}}\right) = \frac{\text{Neat Area}}{t(\text{s}) \times I_{\gamma} \times \varepsilon \times M(\text{kg})} \quad (1)$$

Neat Area is the net area of the photon peak minus the background radiation peak, t is the calculated time (3600 seconds), I_{γ} is the gamma probability, ε is the efficiency of the NaI(Tl)

detector per gamma energy of the radionuclides detected in the soil, and M is the mass of the soil sample in kilograms.

3.2 Calculation the Hazard Index by Radiation:

The following gamma radiation parameters (radiation hazard indices) were determined:

3.2.1 Annual Effective Dose Equivalent (AEDE):

Equations (2), (3) and (4) are used to determine the annual effective dose equivalent (AEDE) for indoor, outdoor, and total exposures respectively, as shown below [13]:

$$AEDE_{\text{indoor}} \left(\frac{\text{mSv}}{\text{y}} \right) = D_Y \left(\frac{\text{nGy}}{\text{hr}} \right) \times 8760 \left(\frac{\text{hr}}{\text{y}} \right) \times 0.7 \left(\frac{\text{Sv}}{\text{Gy}} \right) \times 0.8 \times 10^{-6} \quad (2)$$

$$AEDE_{\text{outdoor}} \left(\frac{\text{mSv}}{\text{y}} \right) = D_Y \left(\frac{\text{nGy}}{\text{hr}} \right) \times 8760 \left(\frac{\text{hr}}{\text{y}} \right) \times 0.7 \left(\frac{\text{Sv}}{\text{Gy}} \right) \times 0.2 \times 10^{-6} \quad (3)$$

$$AEDE_{\text{total}} \left(\frac{\text{mSv}}{\text{y}} \right) = AEDE_{\text{indoor}} \left(\frac{\text{mSv}}{\text{y}} \right) + AEDE_{\text{outdoor}} \left(\frac{\text{mSv}}{\text{y}} \right) \quad (4)$$

3.2.2 Excess Life Time Cancer Rate (ELCR):

The following mathematical formula can be used to express the lifetime excess cancer incidence rate (ELCR).:

$$ELCR = AEDE_{\text{total}} \times D_L \times R_F \quad (5)$$

Here, RF represents the conversion factor, and DL represents life expectancy, which is generally believed to be around 70 years. The International Commission on Radiological Protection (ICRP) uses a standard value for RF of 0.05 Sv^{-1} for the general population [14, 15].

3.1.3 Internal Hazard Index (Z_{int}):

To determine Internal Hazard Index (Z_{int}) from equation (6) [16].

$$Z_{\text{int}} = \frac{S_{\text{Ra}}}{185} + \frac{S_{\text{Th}}}{259} + \frac{S_{\text{K}}}{4810} \quad (6)$$

3.1.4 External Hazard Index (Z_{ext}):

The Using equation (7), to determine External Hazard Index (Z_{ext}) [17].

$$Z_{\text{ext}} = \frac{S_{\text{Ra}}}{370} + \frac{S_{\text{Th}}}{259} + \frac{S_{\text{K}}}{4810} \quad (7)$$

3.1.5 Index of Gamma Representative (I_{γ}):

Equation (8) can be used to calculate the gamma representation index (I_{γ}) [18,19].

$$I_{\gamma} = \frac{S_{\text{Ra}}}{150} + \frac{S_{\text{Th}}}{100} + \frac{S_{\text{K}}}{1500} = 1 \quad (8)$$

3.1.6 Gamma Rate of Dose Absorption (D_Y):

To obtain the gamma dose absorption rate (D_Y) can be used Equation (9) [20, 21].

$$D_Y (\text{nGy h}^{-1}) = 0.462 S_{\text{Ra}} + 0.621 S_{\text{Th}} + 0.0417 S_{\text{K}} \quad (9)$$

3.1.7 Radium Equivalent Activity (R_{eq}) :

A measurement called radium equivalent radioactivity (R_{eq}) is used to determine the total radioactivity of the radium isotopes present in a sample. The specific activity of radium-226 ($Ra-226$), a radioactive isotope of radium, is used to calculate the R_{eq} value. Using Equation (10), this measurement helps assess the potential radiological hazards associated with materials containing radium and its decay products, specifically $Ra-226$ ($U-238$), A_{Th} for $Th-232$ and A_K for $K-40$. [22]

$$R_{eq} \left(\frac{Bq}{kg} \right) = S_{Ra} + 1.43S_{Th} + 0.077S_K \quad (10)$$

4. Results and Discussion

Table 3 represents the Specific activity values (Bq/kg) in soil samples for radionuclides: $Ac-228$ (969 keV), $Cs-137$ (662 keV), $Ra-226$ (186 keV), $K-40$ (1460keV) and $Bi-214$ (609 keV).

Table 3- Specific activity values (Bq/kg) in soil samples for radionuclides: $Ac-228$ (969 keV), $Cs-137$ (662 keV), $Ra-226$ (186 keV), $K-40$ (1460keV) and $Bi-214$ (609 keV).

Code	Specific Activity for Radionuclides (Bq/kg)				
	Ac-228 969 keV	Cs-137 662 keV	Ra-226 186 keV	K-40 1460keV	Bi-214 609 keV
W1	75.68	16.81	9.46	154.6	214.67
W2	26.36	17.48	23.16	263.31	26.91
W3	0	5.16	4.80	172.81	7.95
W4	72.87	15.34	8.86	1716.01	23.61
W5	28.91	12.21	77.11	409.06	155.93
N1	0	9.64	33.20	137.29	16.33
N2	177.97	3.82	25.50	262.85	48.73
N3	79.64	16.35	0	192.04	25.16
N4	69.54	14.48	0	116.52	185.04
N5	0	16.81	68.29	180.77	214.73
Mean	54.11	12.81	25.04	381.38	91.01
Acceptable Limit UNSCEAR (2010) [23]	-----	-----	-----	412	-----

According to Table 3, sample W3 recorded the lowest specific radioactivity for $Th-232$ ($Ac-228$) at (0) Bq/kg. At the University of Baghdad, sample N2 recorded the highest specific radioactivity at 177.97 Bq/kg, while the average specific radioactivity for all samples was (54.11) Bq/kg. Except for sample W4 (1716.10 Bq/kg), which recorded the highest specific radioactivity, the lowest specific radioactivity for $K-40$ (Bq/kg) for all samples was below the

permissible limit. The average value of K-40 for all samples was 381.38 Bq/kg, also samples (N3 and N4) from outside the University of Baghdad recorded the lowest specific radioactivity for Ra-226 (0) Bq/kg, while sample (W5) from within the University of Baghdad recorded the highest specific radioactivity (77.11) Bq/kg. The average specific radioactivity for all samples was (25.04) Bq/kg.

Figure 2 illustrates the relationship between the average levels of radioactive elements in samples taken from within and outside the University of Baghdad. The difference is observed to be variable and below the permissible limit, although potassium registers the highest level due to the use of certain types of fertilizers rich in this radioactive element.

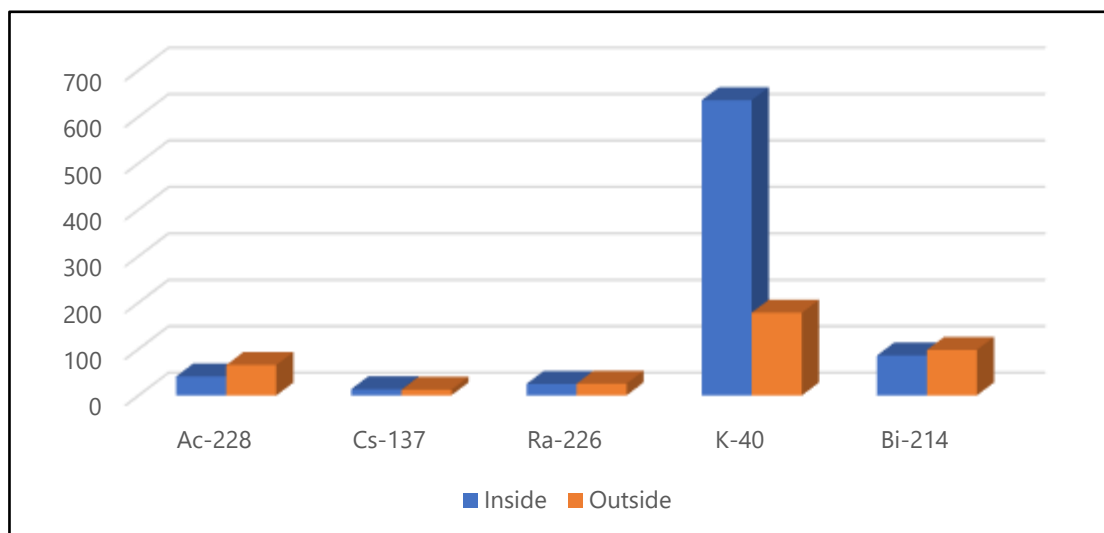


Figure 2: The difference between the average values of radioactive elements inside and outside the University of Baghdad.

Table 4 represents The values of hazard index in soil samples $ELCR \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{in} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{out} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{total} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, H_{in} , H_{ex} , I_{γ} , $D_{\gamma} \text{ (nGyh}^{-1})$ and $Ra_{eq} \text{ (Bq/kg)}$.

Table 4- The values of hazard index in soil samples $ELCR \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{in} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{out} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, $AEDE_{total} \times 10^{-3} \left(\frac{Sv}{Gy} \right)$, H_{in} , H_{ex} , I_{γ} , $D_{\gamma} \text{ (nGyh}^{-1})$ and $Ra_{eq} \text{ (Bq/kg)}$

Code	$AEDE_{in}$ $10^{-3} \left(\frac{Sv}{Gy} \right)$	$AEDE_{out}$ $10^{-3} \left(\frac{Sv}{Gy} \right)$	$AEDE_{total}$ $10^{-3} \left(\frac{Sv}{Gy} \right)$	ELCR 10^{-3}	Z_{in}	Z_{ex}	I_{γ}	D_{γ} $(nGyh^{-1})$	Ra_{eq} Bq/kg
W1	0.251	0.063	0.313	1.095	0.34	0.32	0.82	51.06	116.987
W2	0.133	0.033	0.179	0.581	0.23	0.16	0.42	27.07	60.866
W3	0.011	0.002	0.014	0.047	0.03	0.01	0.03	2.22	4.804
W4	0.638	0.161	0.798	2.794	0.68	0.66	1.93	130.21	245.257

W5	0.632	0.158	0.791	2.770	0.86	0.65	1.87	128.98	149.948
N1	0.085	0.021	0.094	0.331	0.18	0.09	0.22	15.34	33.196
N2	0.599	0.153	0.751	2.625	0.82	0.75	1.95	122.29	279.981
N3	0.242	0.061	0.303	1.061	0.31	0.31	0.79	49.46	113.897
N4	0.238	0.060	0.298	1.041	0.30	0.30	0.78	48.52	111.737
N5	0.155	0.041	0.193	0.683	0.37	0.18	0.46	31.55	68.293
Mean	0.303	0.087	0.381	1.325	0.42	0.35	0.94	61.74	128.867
Acceptable Limit UNSCEAR (2000) [6]	-----	-----	0.083×10^{-3}	0.29×10^{-3}	≤ 1	≤ 1	1	-----	370

The largest indoor yearly dynamic dose equivalent was (638×10^{-6}) mSv/y, while the lowest rate was (11×10^{-6}) mSv/y, with an average ratio of (303×10^{-6}) mSv/y, according to the indoor annual effective dose equivalent (**AEDE**) obtained by applying equation (2). Equation (3) was used to get the outdoor annual effective dose equal (**AEDE**). The average value was (87×10^{-6}) mSv/y, with the greatest rate being (161×10^{-6}) mSv/y and the lowest rate being (2.00×10^{-6}) mSv/y.

The (**ELCR** $\times 10^{-3}$) values varied from 47 to 2794, with an average of 1325. The external hazard index (**Z_{ex}**), which was obtained by using equation (7), had an average value of 0.35 and a maximum value of 0.75, which is greater than the permitted value of 1.00. The lowest value was 0.01. The internal hazard index (**Z_{in}**), which was calculated using equation (6), has an average value of 0.42, with the highest value being 0.86 and the lowest value being 0.03.

Soil radionuclide concentrations determine the contribution of naturally occurring radionuclides to the atmospheric absorption dose, as represented by the gamma index. Soil radionuclide concentrations are directly related to terrestrial gamma radiation. Equation (8) can be used to determine the role of terrestrial gamma-ray radiation in atmospheric absorption doses. The highest value of the representative gamma index (**I_y**) obtained by applying Equation (8) was 1.95. In contrast, the average value of the representative gamma index was 0.94, while the lowest value was 0.03. Equation (9) is used to determine the gamma ray dose absorption rate (**D_y**). The highest gamma ray dose absorption rate was 130.21 nGy/h, while the lowest was 2.22 nGy/h, with a mean of 61.74 nGy/h. Equation (10) explains how to calculate radium (**R_{aeq}**). Sample (N2) outside the University of Baghdad recorded the highest radium equivalent activity value of 279.98 Bq/kg, which is below the permissible value of 370 Bq/kg. Sample (W3) inside the University of Baghdad recorded the lowest radium equivalent activity value of 4.80 Bq/kg, with a mean of 128.86 Bq/kg.

Figure 3 shows the average radiation hazard index values between samples inside and outside the University of Baghdad, and the highest average was found for samples inside the university.

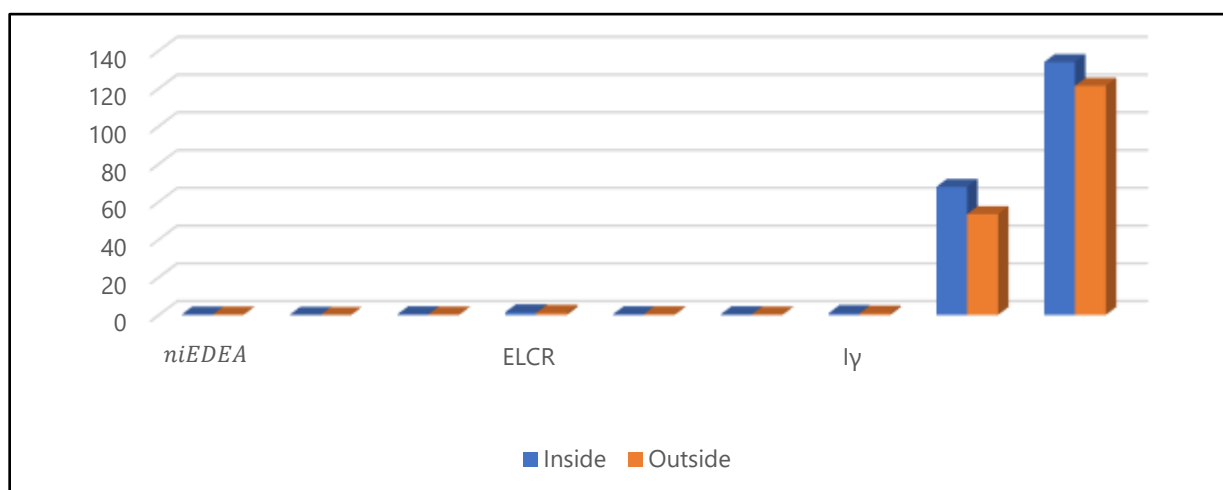


Figure 3: The average values of the radiation hazard index calculated for samples inside and outside the University of Baghdad.

5. Conclusions

1. With the exception of a notable increase in potassium-40 concentration in sample W4, the results show variation in radioactivity levels among all samples, with the majority of values falling within acceptable limits. The average concentrations indicate a moderate level of radioactivity in the environment overall.

2. Thorium-232, radium-226, and potassium-40 levels vary but are generally acceptable when comparing samples taken from within and outside the University of Baghdad. Potassium is the most significant contributor, likely due to fertilizer use.

3- The investigation results indicated that radiation exposure levels were generally low, but varied geographically. Indoor values ranged from 11 to 638×10^{-6} mSv/year, while outdoor values ranged from 2.00 to 161×10^{-6} mSv/year.

4- With only a few elevated readings (such as Iy up to 1.95 and Zex near 0.75), the radiation hazard indices (ELCR, Zex, Zin, Iy, Dy, and Raeq) remained mostly within acceptable limits, indicating generally safe conditions with limited areas of higher radiation exposure.

5. Regarding the activity of naturally occurring radionuclides and radiation hazard indicators, all soil samples tested fall within the internationally accepted safe range. However, one sample (W4) showed relatively high values, and the relevant authorities recommend further research and monitoring.

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