

# Multifunctional MWCNT/NiWO<sub>4</sub>/ZIF-8 Nanocomposite: Structural Characterization, Antibacterial Activity, Cytocompatibility and Cytokine-Modulating Response

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## Research Article

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**Multifunctional MWCNT/NiWO<sub>4</sub>/ZIF-8 Nanocomposite: Structural Characterization, Antibacterial Activity, Cytocompatibility and Cytokine-Modulating Response**

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## Abstract

Nanocomposites with complex structure that provide antimicrobial and biological activities are gaining growing attention as a novel platform for biomedical and functional surface materials applications. In this study, hierarchical MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite has been prepared via step-wise assembly of multiwalled carbon nanotubes (MWCNTs), nickel tungstate (NiWO<sub>4</sub>) and zeolitic imidazolate framework-8 (ZIF-8). Structural, morphological, vibrational, optical and surface chemical analysis has been carried out with X-ray diffraction, transmission electron microscopy, Fourier transform-infrared spectroscopy, Raman spectroscopy, ultraviolet-visible diffuse reflectance spectroscopy and X-ray photoelectron spectroscopy. Analysis results have shown co-existence of MWCNT, monoclinic NiWO<sub>4</sub> and crystalline ZIF-8 phases along with intimate interface coupling. Antimicrobial activity of the hierarchical nanocomposite has been evaluated as a function of concentration by zone inhibition test. It has shown growth inhibition up to 13 and 12 mm against *Staphylococcus aureus* and *Enterococcus faecalis*, respectively, at 100 µL. Biological evaluation has been performed by egg albumin denaturation test. The inhibitory activity has been increased from 43% at 25 µg/mL to 92% at 150 µg/mL, reaching closer values of diclofenac response. Further biological activities of the nanocomposite were explored through concentration-dependent cytocompatibility and cytokine-related assays. As a result, the combination of conductive MWCNT, NiWO<sub>4</sub>, and porous ZIF-8 forms a multifunctional nanocomposite with biologically detectable antibacterial and anti-inflammatory activities.

**Keywords:** MWCNT/NiWO<sub>4</sub>/ZIF-8; Hierarchical nanocomposite; Antibacterial activity; Cytocompatibility; Cytokine.

## 1. Introduction

The widespread use of microorganisms and resistance of microbes against antimicrobials has led to the design and fabrication of nanostructured materials that exhibit different physicochemical properties compared to traditional antimicrobials[1]. The unique physicochemical features of nanomaterials, such as high surface-to-volume ratios, controllable surface chemistry, and various contact sites with microbes, can facilitate their biological applications. Carbon-based nanomaterials have received much attention due to their tailorable morphologies, electrical properties, and surface functionalities for biological purposes[2]. Multi-walled carbon nanotubes (MWCNTs), comprising concentric graphite carbon layers, have a high aspect ratio, extensive  $\pi$ -electronic system, conductivity, and accessible surface areas. These properties of MWCNTs allow them to come into close contact with microbial cell walls and also act as conductive templates for inorganic functional phases. However, agglomeration of nanotubes and morphological, surface, functionalization, and dosing dependent biological responses may affect their independent usage in biological applications. Hence, the combination of MWCNTs with inorganic and porous nanostructures is a logical approach[3,4].

The chemical properties of MWCNTs offer further possibilities for adding antimicrobial properties. The long  $\pi$ -electronic system enables electron transfer processes at interfaces, defect-prone areas and surface polar functional groups like hydroxyl, carbonyl, carboxyl groups can change the surface polarity, wettability, charge distribution and interaction with microbial membranes. The functionalization may also help to disperse MWCNTs in aqueous or polymeric media to increase the proportion of nanotube surface available for interactions with biological systems. It is generally believed that the antimicrobial effect of MWCNTs is based on physical and chemical effects, not on one only. The contact force between the nanotube and the bacterial membrane may result in local mechanical stress and

nanoscale damage to the membrane, while alteration in the surface chemistry of the nanotube may lead to oxidative processes and disruption of the cells' redox balance. Overabundant generation of reactive oxygen species (ROS) may lead to lipid peroxidation, protein oxidation, DNA damage, and impairment of key metabolic pathways[5]. Indeed, the small diameter of MWCNTs combined with the high aspect ratio means that the chances of contact with microorganisms will be higher and that they may be able to wrap or trap bacterial cells within nanotube networks. Therefore, MWCNTs can act to cause physical damage to the membrane, to have adsorption sites, and to have an electronically conducting pathway for charge transport. The tendency of nanotubes to form aggregates, however, can reduce the effective surface area, and limit the antimicrobial activity. This can be overcome by combining MWCNTs with inorganic materials and porous materials to form a hybrid material, while MWCNTs form a continuous network of structure and conductivity, the inorganic and porous materials can prevent excessive agglomeration and give MWCNTs more antimicrobial functions. From this point of view, it is noteworthy that nickel tungstate ( $\text{NiWO}_4$ ) was incorporated, as transition-metal tungstates have unique electronic, catalytic and surface-redox properties[6].

Nickel tungstate ( $\text{NiWO}_4$ ) provides a complementary inorganic component for constructing such hybrid architectures.  $\text{NiWO}_4$  is a transition-metal tungstate with a metal–oxygen framework and surface-active Ni and W centers that can participate in interfacial charge-transfer and redox processes. Previous studies have explored  $\text{NiWO}_4$ -based materials for photocatalytic, electrochemical, sensing, and antimicrobial applications. When integrated with a conductive carbon framework, the interfacial electronic properties of  $\text{NiWO}_4$  may be modified through improved charge transport and dispersion of the inorganic phase. Thus, incorporation of  $\text{NiWO}_4$  onto MWCNTs can potentially combine the conductive and high-aspect-ratio characteristics of the carbon framework with the surface chemistry of the tungstate phase. Such interfacial organization may increase the accessibility of inorganic active sites while reducing the limitations associated with aggregation of individual components[7]. The

antimicrobial activity of  $\text{NiWO}_4$  can be achieved by the generation of oxidative stress as a result of surface-mediated reactions, especially when the  $\text{NiWO}_4$  is paired with a suitable conductive material. The integration of  $\text{NiWO}_4$  with MWCNTs is expected to give a positive interfacial relationship: MWCNT network can help to transport electrons and  $\text{NiWO}_4$  provides transition metal oxide sites which can participate in redox reactions. The efficient charge migration at the MWCNT/ $\text{NiWO}_4$  interface can decrease the recombination of charge carriers and enhance the lifetime of the reactive species at the material–solution interface. These oxidative species may react with microbial cell walls and membranes leading to disruption of cell membrane lipid and protein oxidation, as well as to the disturbance of the intracellular homeostasis. Besides, the conductive MWCNT framework can enhance the dispersion of  $\text{NiWO}_4$  nanocrystals, enhance the surface area of the nanocrystals, and create more contact points with microbial cells. However, the use of MWCNT and  $\text{NiWO}_4$  separately may not take full advantage of the potential of a multifunctional antimicrobial surface[8,9]. The hybrid system also has an additional chemical and structural dimension as a result of incorporation of a porous metal–organic framework, zeolitic imidazolate framework-8 (ZIF-8)[10].

Zeolitic imidazolate framework-8 (ZIF-8) introduces a third functional dimension through its porous metal–organic framework architecture. ZIF-8 consists of  $\text{Zn}^{2+}$  nodes coordinated by 2-methylimidazolate linkers and is characterized by a porous structure, high surface area, and tunable surface chemistry. These properties have led to considerable interest in ZIF-8-based materials for adsorption, catalysis, sensing, drug delivery, and antimicrobial applications. The Zn-containing framework may additionally contribute to antimicrobial responses through interactions of Zn-containing sites with microbial membranes and biomolecules, although the magnitude and mechanism of such effects depend on the physicochemical environment and material composition. In a ternary architecture, ZIF-8 may also provide a porous support for the inorganic phase and increase the number of accessible interfacial sites. Its combination with MWCNTs and  $\text{NiWO}_4$  therefore offers an opportunity to

integrate conductivity, transition-metal tungstate chemistry, and porous framework functionality within a single nanocomposite[11,12]. The antimicrobial aspect of the Zn-containing coordination framework is of particular interest as ZIF-8 can release biologically active  $\text{Zn}^{2+}$  as a result of the degradation of the framework or due to the release of ions under suitable environmental conditions. Zinc ions may bind with cell membranes and biomolecules and inhibit enzyme activity, disrupt membrane permeability and interfere with metabolic processes in bacteria[13]. ZIF-8 can thus provide a chemical antimicrobial pathway, in addition to the physical pathway of membrane interactions of MWCNTs and redox pathway of  $\text{NiWO}_4$ . It can also have a porous structure, which can help to adsorb the microbial cells or biomolecular species and offer a large number of accessible interfacial sites[14,15]. More significantly, ZIF-8 can be a functional nanostructured support for  $\text{NiWO}_4$  which can reduce aggregation of particles and enhance the distribution of inorganic active sites. The porous framework can be attached to the conductive carbon network, forming a hierarchy with multiple length scales and chemically different interfaces when combined with MWCNTs. This type of structure may improve surface accessibility and lead to close contact between microorganisms and active ingredients.  $\text{Zn}^{2+}$ -mediated toxicity, oxidative stress and physical membrane disruption may create a more potent antimicrobial activity than can be achieved through any single one of these. Therefore, the rational combination of ZIF-8 with MWCNTs and  $\text{NiWO}_4$  represents a chance to develop an antimicrobial material, not a material consisting of two parts that work synergistically by the same mechanism. Thus, the rational combination of ZIF-8 with MWCNTs and  $\text{NiWO}_4$  offers a chance to develop an antimicrobial material, not a material consisting of two parts that work synergistically by the same mechanism[16,17].

Although MWCNT-,  $\text{NiWO}_4$ -, and ZIF-8-based materials have individually and in various binary combinations been investigated for functional and biological applications, the integration of these three components into a single MWCNT/ $\text{NiWO}_4$ /ZIF-8 architecture provides a distinct combination of conductive carbon, transition-metal tungstate, and porous

metal–organic framework characteristics. In particular, systematic evaluation of the structural integration of these phases together with their antibacterial response, cell viability, and inflammation-related biological responses remains limited. The biological performance of such a ternary system cannot be assumed solely from the properties of its individual constituents because interfacial organization, phase dispersion, surface chemistry, and composition can substantially influence the resulting response. Therefore, experimental evaluation of the ternary architecture is necessary to establish whether the combined material provides measurable biological functionality while maintaining acceptable cellular viability within the investigated concentration range[18–20]. This antimicrobial response can include adsorption and mechanical disruption of the cell envelope, changes in membrane permeability, ROS-induced oxidative damage, peroxide-induced cellular stress, metal-ion induced cellular stress and interference with essential metabolic functions[21]. This multi-modal antimicrobial response is especially relevant considering that microbes are less likely to adapt to multiple, at the same time, encountered physicochemical stresses in a single adaptive response. However, the effectiveness of the hybrid relies greatly on the structural organization of the components, their interfacial contact, the surface chemistry, and the dispersion of the components. Thus, the study of the antimicrobial activity of MWCNT/NiWO<sub>4</sub>/ZIF-8 is not only crucial to determining whether it has microbial inhibitory properties, but also for understanding the influence of the synergistic effect of the three components, MWCNT, NiWO<sub>4</sub>, and ZIF-8, on biological activity. The present study consequently focuses on the antimicrobial activity of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite and explores its potential as a multifunctional nanostructured antimicrobial material. The use of high surface area carbon, transition metal tungstate chemistry and porous zinc based framework architecture is a promising platform for the design of improved antimicrobial systems that have enhanced interfacial activity and potentially broad-spectrum microbial inhibition.

## 2. Materials And Methods

### 2.1. Materials

Nickel nitrate hexahydrate ( $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 99.9%), sodium tungstate dihydrate ( $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ , 99.9%), zinc nitrate hexahydrate ( $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ , 98.0%, Sinopharm), 2-methylimidazole (2-MeIm, 99.0 %, Sigma-Aldrich), dimethyl sulfoxide (DMSO), multi-walled carbon nanotubes (MWCNTs), ethanol ( $\text{C}_2\text{H}_5\text{OH}$ ), potassium hydroxide (KOH), nickel foam, and distilled water served as starting materials and solvents for synthesis and subsequent analyses. All chemicals and reagents were of analytical grade and used as received without further purification. We purchased chemicals from Sigma-Aldrich and used them throughout the experimental procedures. Distilled water was utilized for solution preparation and washing steps to minimize the influence of extraneous ionic impurities.

### 2.2. Preparation of ZIF-8

ZIF-8 was prepared with the organic linker 2-methylimidazole and metal precursor zinc nitrate hexahydrate. In brief, 1.242 g of 2-methylimidazole was weighed accurately and dissolved in 25 mL of N,N-dimethylformamide (DMF). This solution was then continually stirred for 30 min at room temperature to ensure a homogeneous solution of ligands. 1.487 g of zinc nitrate hexahydrate [ $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ] was dissolved in 25 mL of DMF while stirring for 30 min to ensure complete dissolution of the metal salt in the medium. Zinc precursor solution was then poured into the solution of the 2-methylimidazole with continuous stirring. The resulting reaction mixture was stirred for 1 h to ensure even coordination of the deprotonated 2-methylimidazole with the  $\text{Zn}^{2+}$  ions. The homogeneous solution was kept in the dark for 42 h at room temperature. The aging step gives enough time for the nucleation and crystal growth of the ZIF-8 framework to occur, allowing the coordination reaction and the formation of the porous crystalline structure to take place. After the aging process, the reaction mixture was moved into stainless-steel autoclave with a Teflon liner and the solvothermal treatment was

carried out at 140 °C for 6.5–7 h. The high temperature favours the formation and crystallization of the Zn–2-methylimidazolate framework. Once the solvothermal process was finished, the autoclave was left to cool down naturally to room temperature. The solid product was separated via filtration and washed three times with DMF to remove unreacted organic ligand, loosely bound species and soluble reaction by-products. The dried washed precipitate was then dried for 48 h at 60 °C to yield the ZIF-8 powder. The retrieved material was kept in a clean dry container for future use.

### **2.3. Preparation of Nickel Tungstate (NiWO<sub>4</sub>)**

A controlled aqueous precipitation process was employed to synthesize nickel tungstate with nickel(II) nitrate hexahydrate and sodium tungstate as the nickel and tungsten source, respectively. A solution of 0.5815 g of Ni(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O in 20 mL of distilled water was prepared, which resulted in a 0.1 M nickel precursor solution. The solution was magnetically stirred for 15 min at room temperature to assure the complete dissolution and homogeneous distribution of Ni<sup>2+</sup> ions. This solution became a solution called Solution A. The sodium tungstate was dissolved in 20 mL of distilled water in a separate beaker to make a 0.1 M tungstate precursor solution. Solution B was then slowly added to Solution A, while continuously stirring, to get a homogeneous mixture and was labelled as Solution B. By using the controlled addition of the tungstate precursor, the concentration gradients were minimized in the immediate vicinity and the interactions between Ni<sup>2+</sup> and tungstate species were made more homogeneous. The mixture was stirred continuously for 1 h at the room temperature, which led to the formation of the nickel–tungstate precursor phase and to a better compositional uniformity and was designated as Solution C. The precipitation process can be generally described in terms of the reaction of Ni<sup>2+</sup> ions with tungstate species to form the framework of nickel tungstate:  $\text{Ni}^{2+} + \text{WO}_4^{2-} \rightarrow \text{NiWO}_4$

This nickel tungstate suspension was later used as the nickel tungstate source in the synthesis of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite. It is important to the stage to keep the concentration of the precursors low and the mixing process continuous to achieve the uniformity of the distribution of Ni and W species and to reduce the presence of unwanted secondary phases.

#### **2.4. Preparation of MWCNT/NiWO<sub>4</sub>/ZIF-8 Hybrid Composite**

The MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite was prepared by sequential dispersion, interfacial assembly and solvothermal methods. In brief, 0.150 g of commercial MWCNTs was dispersed into 20 mL of distilled water using magnetic stirring for 20 min and then sonication for 5 min to enhance the dispersion of the nanotubes. Then 0.5 g of ZIF-8 synthesized was dispersed in 20 mL of distilled water under stirring for 20 min and sonicated for 5 min separately. Then, the MWCNTs suspension was gently added to the ZIF-8 dispersion while stirring to ensure the uniform interfacial contact. Then a pre-prepared nickel–tungstate precursor (Solution C) was dropwise added while continuously stirring. The resulting suspension was stirred 3 h at room temperature and sonicated for 5 min, to ensure homogeneity and to reduce aggregation. The well dispersed suspension was then transferred to a Teflon lined stainless-steel autoclave and treated under solvothermal conditions at 150 °C for 8 h. This process allowed the formation and stabilization of NiWO<sub>4</sub> and intimate integration between the three components. The sample was then allowed to cool naturally and then filtered and washed many times with distilled water, ethanol and acetone to wash out the residual salts and unreacted species. The MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite was obtained after drying the purified product for 48 h at 60 °C.

#### **2.5. Characterizations**

The synthesized MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was characterized by various complementary techniques to study their structural, morphological, vibrational, optical and

surface-chemical properties. The crystalline phases and the structural characteristics of the samples were analyzed by powder X-ray diffraction (XRD) using a Bruker D8 Advance diffractometer with monochromated Cu K $\alpha$  radiation ( $\lambda = 1.5406 \text{ \AA}$ ). The Raman spectrometer is a Thermo Fisher H31XYZE, and the carbon structural features, degree of graphitization and defect characteristics were analyzed using this Raman spectrometer. The characteristic vibrational modes and surface functional groups of the synthesized material were identified using Fourier-transform infrared (FTIR) spectroscopy. The optical absorption properties were studied in the range of wavelength (200 – 800 nm) with Shimadzu Uv-1800 UV-visible spectrometer and optical band-gap characteristics were determined from the obtained absorption data. X-ray photoelectron spectroscopy (XPS) measurements were performed on a Physical Electronics PHI VersaProbe II spectrometer using monochromatic Al K $\alpha$  radiation (1486.6 eV, 15 kV), a dual beam charge neutralizer and a hemispherical multichannel detector. The XPS spectra were measured at a fixed pass energy of 29.35 eV with a beam diameter of 100  $\mu\text{m}$ , a step size of 0.125 eV and standing time of 10 s. The obtained spectra were processed and analyzed by CasaXPS software to obtain the elemental composition and various chemical states of the constituent elements.

## **2.6. Antibacterial assay**

The synthesized MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was tested for its antimicrobial activity against *Staphylococcus aureus* and *Enterococcus faecalis* using agar well diffusion method. In brief, a homogeneous bacterial lawn was prepared by uniformly inoculating freshly prepared bacterial cultures onto sterile nutrient agar plates. A sterile well borer was then used to form wells with a uniform diameter in the inoculated agar. The synthesized MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was suspended in sterile-distilled water (100  $\mu\text{g/mL}$ , 1 mg in 10 mL) and different amounts of it (50, 75 and 100  $\mu\text{L}$ ) were sequentially added into the designated wells. Sterile distilled water was used for the negative control and the

appropriate antibacterial standard was used for the positive control. The plates were then incubated for 24 hours at 37 °C under appropriate conditions. After incubation the inhibition of the microorganisms was evaluated by measuring the zone of inhibition around each well with a ruler or digital measurement tool. The size of the inhibition zone was taken as an indication of suppression of bacterial growth as a larger clearance indicated more antibacterial activity. The experimental conditions for all the treatments and controls were kept the same to provide reliable comparison of the antibacterial response of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite with the selected bacterial strains.

## **2.7. Antioxidant Activity — DPPH Radical-Scavenging Assay**

The ability of synthesized MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite to act as an antioxidant was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. Freshly prepared solution of DPPH was prepared in methanol under the condition of absence of light. Nanocomposites were diluted to prepare the concentrations of 25, 50, 75, 100, 125 and 150 µg/mL. Each one was then mixed with the DPPH solution and kept in the dark for some time at room temperature. Ascorbic acid served as reference antioxidant while the DPPH solution alone was taken as control. The absorbance was measured at 517 nm using UV-visible spectrophotometer. The experiment was carried out thrice, and the values were given as mean ± SD. Percentage of DPPH radical scavenging activity was determined by formula: DPPH scavenging activity (%) =  $(A_c - A_s) / A_c \times 100$ , where  $A_c$  is the absorbance of the control and  $A_s$  is the absorbance of the sample.

## **2.8. Cell Viability and Cytotoxicity Assay**

The cytocompatibility of MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was tested via MTT assay on RAW 264.7 macrophages. Cells were cultured in 96-well plates at an optimal cell density and incubated under normal cell culture conditions. The cells were treated with the nanocomposite at various concentrations of 25, 50, 75, 100, 125 and 150 µg/mL for the specified duration.

Cells without treatment served as controls. After incubation, MTT solution was introduced into each well and the metabolically active cells reduced the MTT into insoluble formazan crystals. The media were aspirated out and formazan crystals were solubilized using a suitable solubilization reagent. The absorbance was taken at 570 nm using microplate reader. Cell viability was calculated as the percent of viability compared to the control as follows:  $\text{Cell viability (\%)} = A_c/A_s \times 100$ , where  $A_s$  represents the absorbance of nanocomposite-treated cells and  $A_c$  represents the absorbance of untreated control cells. The experiment was performed using independent replicates, and the results were expressed as mean  $\pm$  SD.

## **2.9. Anti-inflammatory activity**

### **2.9.1 Egg-Albumin Denaturation Assay**

The synthesized MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was tested for anti-inflammatory activity by the egg albumin denaturation test, which was slightly changed from the previously reported procedure. In brief, the reaction mixture was made up of 0.2 mL of fresh egg albumin, 2.8 mL of phosphate buffered saline (PBS, pH 6.4) and 0.6 mL of MWCNT/NiWO<sub>4</sub>/ZIF-8 dispersion at different concentrations. The nanocomposite was examined at the end concentrations, 25 $\mu$ g/mL, 50 $\mu$ g/mL, 75 $\mu$ g/mL, 100 $\mu$ g/mL, 125 $\mu$ g/mL and 150 $\mu$ g/mL in reaction mixture. The nanocomposite was first dispersed in 0.2% DMSO (v/v) to get homogeneity test solutions. The reaction mixtures were shaken gently and allowed to incubate at 37 °C for 10 min and then heated at 70 °C for 20 min in a thermostatically controlled waterbath to induce denaturation of the protein. The mixtures were heated and cooled to the room temperature following which absorbance was recorded at 660 nm in a UV–visible spectrophotometer. A negative control was prepared as for the experimental group but with 2.8 mL of PBS (pH 6.4) in place of the egg albumin and DMSO. The same concentration range was used whereas sodium diclofenac was used as a reference anti-inflammatory standard. All experiments were triplicated and results reported as mean with statistical variations. The

percent inhibition of albumin denaturation was estimated as:  $A_c - A_s / A_c \times 100$ , where  $A_c$  is the absorbance of the control reaction and  $A_s$  is the absorbance of the sample-treated reaction. All measurements were performed in triplicate and expressed as mean  $\pm$  SD.

### **2.8.2 ELISA Analysis of Pro-inflammatory Cytokines**

Anti-inflammatory property of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was additionally investigated in RAW 264.7 macrophages exposed to LPS by quantification of the levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$ . RAW 264.7 macrophages were exposed to LPS in order to induce inflammation and further incubated with different concentrations of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite (25, 50, 75, 100, 125 and 150  $\mu$ g/mL) under the defined experimental conditions. After the treatment, the samples were centrifuged to collect culture supernatants which were further used for measurement of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol. Absorbance was read on a microplate reader at a defined wavelength for each ELISA kit. The concentration of cytokines was determined from the respective standard curve. The experiments were conducted using independent samples and results were presented as mean  $\pm$  standard deviation (SD).

### **2.9. Statistical analysis**

All experiments were performed using independent replicates, and the results were expressed as mean  $\pm$  standard deviation (SD). Statistical significance was evaluated using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test where appropriate. Differences were considered statistically significant at  $p < 0.05$ .

### 3. Results and Discussion

#### 3.1 Structural Characterization

##### 3.1.1 X-ray Diffraction (XRD) Analysis

The X-ray diffraction (XRD) pattern of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite indicates that the three crystalline components are well integrated to retain their characteristic structural features in Figure 1. The low-angle reflections at  $2\theta \approx 7.4^\circ$ ,  $10.4^\circ$ ,  $12.7^\circ$  and  $14.7^\circ$  can be assigned to the (011), (002), (112) and (022) planes of ZIF-8, respectively, consistent with its crystalline sodalite (SOD) topology and JCPDS No. 00-062-1030[22–24]. The strong diffraction peak at  $2\theta \approx 26^\circ$  is mainly attributed to the (002) reflection of graphitic MWCNTs, the higher-order graphite-related reflections confirm the graphitic ordering of the nanotube walls and the strong peak is a characteristic of MWCNTs, typically indexed with the graphite JCPDS No. 41-1487[25–27]. In addition, the reflections appearing at approximately  $23.8^\circ$ ,  $24.9^\circ$ ,  $30.9^\circ$ ,  $31.5^\circ$ ,  $36.4^\circ$ ,  $37.1^\circ$ ,  $39.2^\circ$ ,  $41.7^\circ$ ,  $44.6^\circ$ ,  $46.2^\circ$ ,  $48.9^\circ$ ,  $51.2^\circ$ ,  $52\text{--}54^\circ$ ,  $63^\circ$ ,  $68^\circ$  and  $72^\circ$  are attributable to the monoclinic NiWO<sub>4</sub> phase, corresponding to planes such as (011), (110), ( $-111$ ), (020), (002), (120), (200), ( $-102$ ), ( $-112$ ), ( $-211$ ), (022), (220), (130), ( $-202$ ), ( $-113$ ), ( $-311$ ) and (141), respectively. The reflections are consistent with the monoclinic NiWO<sub>4</sub> structure (P2/c) of NiWO<sub>4</sub> as reported in JCPDS No. 15-0755[28–30]. The absence of significant unidentified impurity peaks indicates high phase purity and successful phase formation for the presence of both ZIF-8 and graphitic MWCNT reflections and NiWO<sub>4</sub> reflection along with the monoclinic phase. The wide background and the lower intensity of some of the individual reflections are reasonably correlated with a nanoscale dimension, with heterogeneous interfaces and partial masking of crystalline domains in the ternary structure.

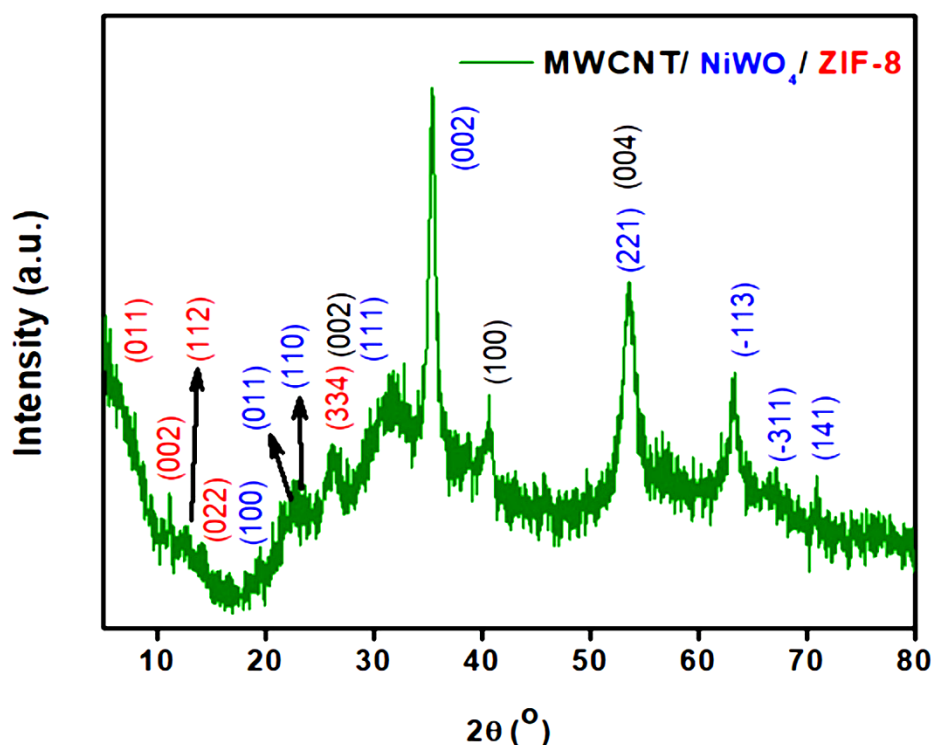


**Figure 1.** X-ray diffraction (XRD) pattern of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite.

### 3.1.2. Fourier Transform Infrared (FTIR) Spectroscopy

The Fourier transform infrared (FTIR) spectrum of the hybrid composite of MWCNTs/NiWO<sub>4</sub>/ZIF-8 offers crucial insights into the retention and incorporation of the inherent chemical functionalities of all three components in Figure 2. The wide band at about 3400 cm<sup>-1</sup> is mainly attributed to the O–H stretching vibration of surface hydroxyl groups and physically adsorbed water molecules. The existence of this band is an indication of the hydrophilic contribution of the surface oxygenated species and surface adsorbed moisture that can be from the aqueous processing and the surface exposed to the hybrid material. The weak absorption bands, located between 2920–2850 cm<sup>-1</sup> could be assigned to C–H stretching vibrations and the band in the 1630 cm<sup>-1</sup> range could belong to the skeletal C=C vibrations of the MWCNT graphitic structure and to the H–O–H bending vibration of water molecules adsorbed on the MWCNT, respectively. ZIF-8 displays several bands in the fingerprint region (between 1600 and 1300 cm<sup>-1</sup>), which correspond to bands of the 2-methylimidazolate linker.

Absorptions at  $\sim 1570\text{--}1500\text{ cm}^{-1}$  can be assigned to C=N stretching modes of the imidazolate ring, and bands at  $\sim 1450\text{--}1350\text{ cm}^{-1}$  are related to C–N stretching, C–C vibrations and ring deformation modes of the coordinated imidazolate ligand. These bands are present even after the incorporation into the ternary composite, suggesting that the organic linker structure is still chemically recognizable. Other absorptions in the  $1300\text{--}1000\text{ cm}^{-1}$  range can be attributed to inplane vibrations and C–N stretching modes of the imidazolate framework, which further confirms the existence of ZIF-8. The lower wavenumber region is of special interest for information in relation to the inorganic  $\text{NiWO}_4$  phase. Absorptions near  $900\text{ cm}^{-1}$  can be assigned to the W–O stretching vibrations of tungstate units and strong absorptions in the  $650\text{--}500\text{ cm}^{-1}$  range are due to the metal–oxygen lattice vibrations including Ni–O and W–O bonds for  $\text{NiWO}_4$  structure. The high intensity of this band, around  $550\text{--}600\text{ cm}^{-1}$ , is therefore important for confirming the formation of the nickel tungstate phase. At the same time, the graphitic carbon framework of MWCNTs brings some vibrational characteristics related to  $\text{sp}^2$ -hybridized carbon, but they could be masked by the more intense bands due to ZIF-8 and  $\text{NiWO}_4$ . Very important, the FTIR spectrum does not show prominent additional bands for the presence of significant amounts of unwanted organic and/or inorganic by-products. The presence of O–H/C–H, C=C, C=N/C–N and Ni–O/W–O related vibrations show that the three components remain in the final hybrid structure. The relative intensity, width and shift of some bands relative to the individual components can be attributed with a certain degree of confidence to the interactions between the phases of  $\text{NiWO}_4$ , MWCNT, and ZIF-8 at the interfaces. These can include surface adsorption, coordination or electrostatic interactions, and electronic coupling at the heterointerfaces. Thus, the FTIR results overall corroborate the successful chemical incorporation of  $\text{NiWO}_4$ , MWCNTs and ZIF-8 and offer additional evidence in addition to XRD analysis of the formation of the targeted ternary hybrid composite.

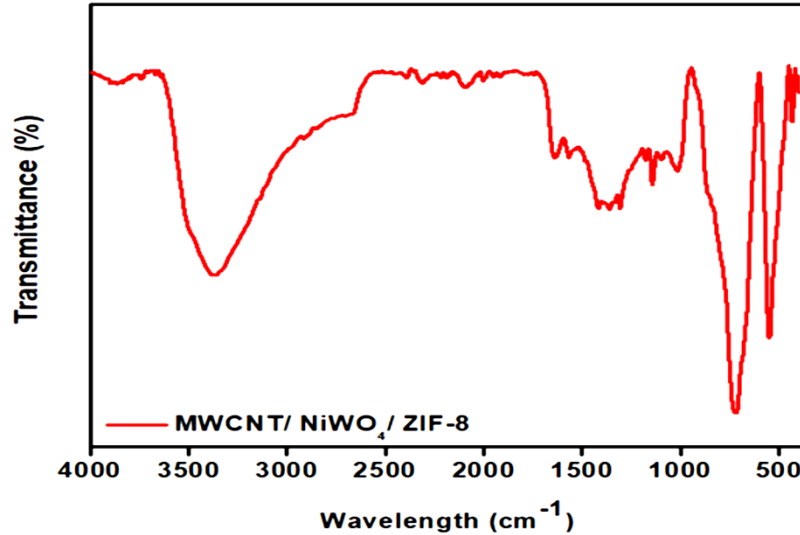


**Figure 2.** FTIR spectrum of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite

### 3.1.3. UV–Visible Diffuse Reflectance Spectroscopy (UV–Vis DRS)

UV–visible diffuse reflectance spectroscopy (UV–DRS) profile of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite is useful for determining the optical absorption characteristics and electronic structure in Figure 3. The composite has a strong absorption in the ultraviolet spectrum below about 350 nm, and a broad, relatively strong absorption over the visible spectrum up to 1100 nm as indicated in the spectrum. The electronic transition in NiWO<sub>4</sub> and ZIF-8 molecules may be responsible for the intensive UV absorption, while the  $\pi$ -conjugated electronic structure of MWCNTs is responsible for the broad absorption towards longer wavelengths. The broad weak maximum may be associated with the electronic transitions of the graphitic carbon framework and the metal–oxygen/organic-linker environments. Also, the broad-band-gap framework has a significantly different optical response than the hybrid, which is particularly significant because of the introduction of states from delocalized  $\pi$ -electrons in MWCNTs and the potential for interfacial electronic interactions with NiWO<sub>4</sub> and ZIF-8. Optical band gap should be tried to be determined quantitatively by the Tauc relation,  $(\alpha h\nu)^n = A(h\nu - E_g)$ , where  $\alpha$  is the absorption coefficient,  $h\nu$  is the photon energy,  $A$  is a proportionality constant and  $n$  depends on the nature of the

electronic transition. A direct allowed transition will use  $n = 2$ , an indirect allowed transition will use  $n = 1/2$ . Based on the absorption edge observed and the characteristic optical response of the  $\text{NiWO}_4$ -containing systems, the  $\text{MWCNT}/\text{NiWO}_4/\text{ZIF-8}$  composite is expected to have an optical band-gap energy in the range of 2.8 eV, but an exact  $E_g$  value should be determined from the intercept of the linear portion of the experimentally constructed Tauc plot and not from the spectrum alone. It is observed that the width of optical absorption band is broadening towards the visible region, indicating that an electronic coupling between MWCNTs and  $\text{NiWO}_4/\text{ZIF-8}$  may occur, which would result in the introduction of additional electronic states and could allow charge transfer across the heterointerfaces. This extended light absorption and interfacial electronic communication can be scientifically important for antimicrobial applications as the mechanism of the photo-induced charge carriers may be involved in surface redox reactions, and play a role in the generation of ROS. Therefore, the UV–DRS outcome suggests the possibility of forming an electronically coupled ternary hybrid with improved optical absorption and a possible improvement in the interfacial charge-transfer characteristics[31].

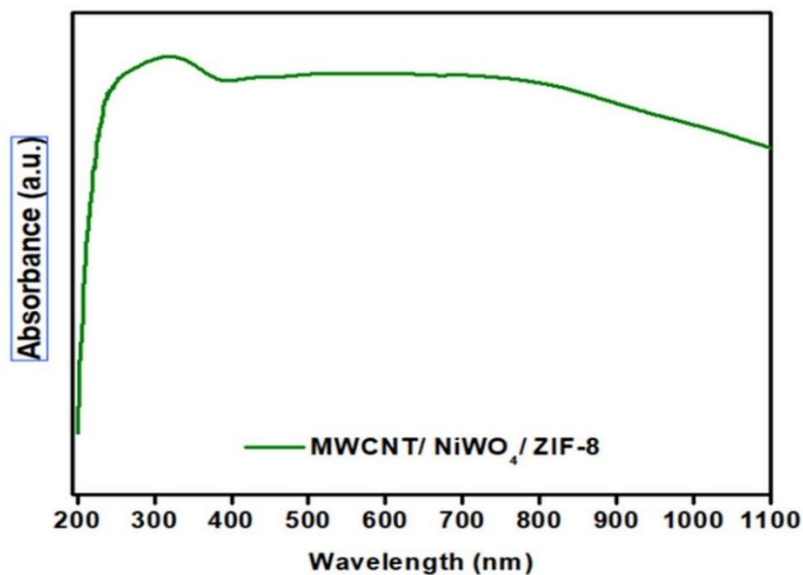


**Figure 3:** UV–visible diffuse reflectance spectrum of the  $\text{MWCNT}/\text{NiWO}_4/\text{ZIF-8}$  nanocomposite

### 3.1.4. Transmission Electron Microscopy (TEM), High-Resolution TEM (HRTEM), SAED

The TEM, HRTEM, and SAED micrographs of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite have been shown in Figure. 4. The TEM micrograph illustrates elongated and curved tubes corresponding to the MWCNT framework with nanoscale domains dispersed within the network. Based on the dispersion of the inorganic/framework phases, it could be inferred that the inorganic phases exist with MWCNT architecture instead of the presence of large aggregated structures. Since there is a relatively heterogeneous contrast in the TEM micrograph due to variation in electron scattering among different phases, phase identification by using the TEM micrograph alone cannot be considered unambiguously. From the HRTEM micrograph, it is possible to identify the lattice fringes with an interlayer distance of around 0.34 nm that corresponds to the graphitic structure of MWCNT. Other lattice fringes present in the decorated regions correspond to crystalline inorganic materials, and the lattice planes have to be indexed only where the d-spacing and micrograph resolution allow it. The SAED micrograph demonstrates diffraction rings and spots, indicating the presence of crystalline/nanocrystalline domains with various orientations[32].

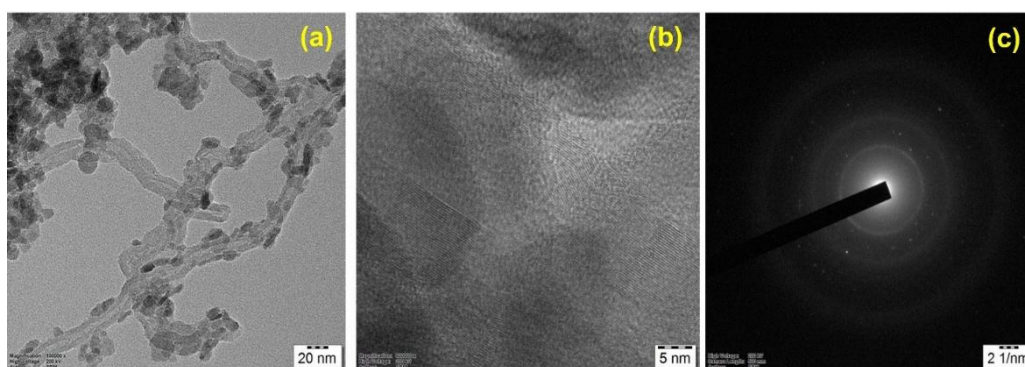


**Figure 4.** TEM, HRTEM and SAED analyses of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid composite: (a) TEM image showing the interconnected MWCNT network decorated with well-dispersed NiWO<sub>4</sub>/ZIF-8 nanodomains, (b) HRTEM image revealing distinct lattice fringes corresponding to the crystalline phases, and (c) SAED pattern exhibiting concentric diffraction rings with discrete spots, confirming the polycrystalline nature and successful integration of MWCNT, NiWO<sub>4</sub> and ZIF-8 within the hybrid composite.

### 3.1.5. Raman Spectroscopic Analysis

Raman spectroscopy was employed to further investigate the structural and vibrational characteristics of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite, and the corresponding spectrum is presented in Fig. 5. The Raman spectrum exhibits bands attributable to the constituent carbon and inorganic/framework components. The bands observed at approximately 326, 474 and 710 cm<sup>-1</sup> can be associated with vibrational modes of the NiWO<sub>4</sub> component, whereas the feature around 984 cm<sup>-1</sup> can be assigned to a W–O-related vibrational mode. Additional bands in the higher-wavenumber region, including those near 1334 and 1581 cm<sup>-1</sup>, are characteristic of the carbonaceous MWCNT framework. The band near 1334 cm<sup>-1</sup> corresponds to the D band and is associated with defect/disorder-related features of the graphitic carbon structure, while the band near 1581 cm<sup>-1</sup> corresponds to the G band and is associated with the in-plane vibration of sp<sup>2</sup>-hybridized carbon. The additional features observed around 1145, 1628 and 1724 cm<sup>-1</sup> may arise from vibrational contributions of the framework and surface functional environments and should be interpreted in conjunction with the FTIR results. The coexistence of the characteristic Raman features of the carbon and inorganic components supports the multiphase nature of the synthesized nanocomposite. Any changes in the relative intensity or position of the D and G bands compared with the starting MWCNTs may indicate changes in the local carbon environment following incorporation of the inorganic/framework phases. Overall, the Raman results complement the XRD and FTIR analyses by providing additional evidence for the structural characteristics of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite[33].

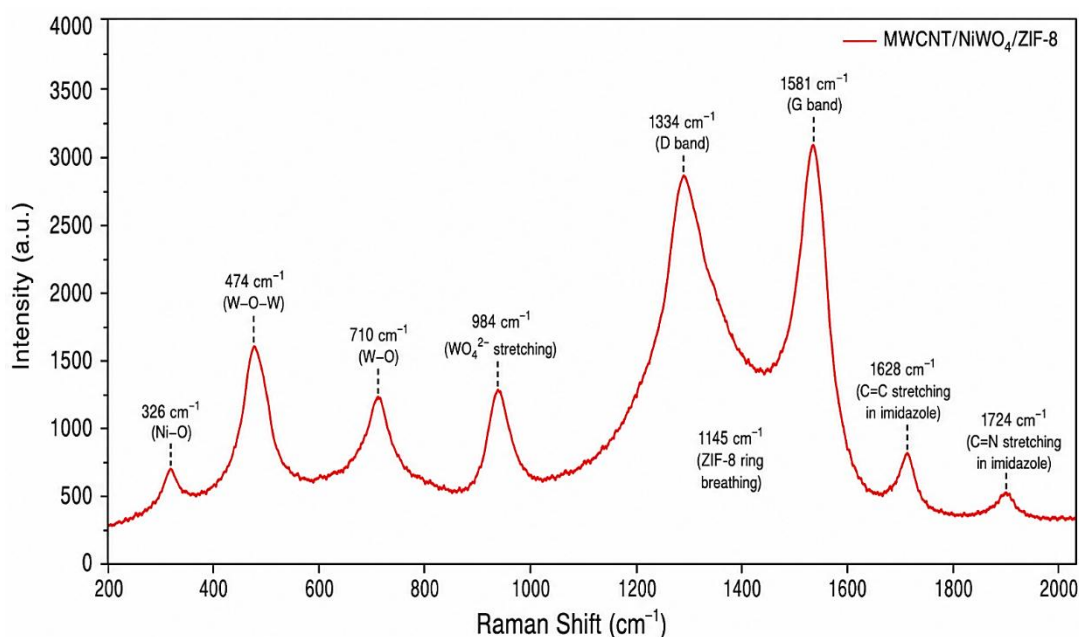


**Figure 5.** Raman spectrum of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite showing characteristic vibrational features of the constituent phases.

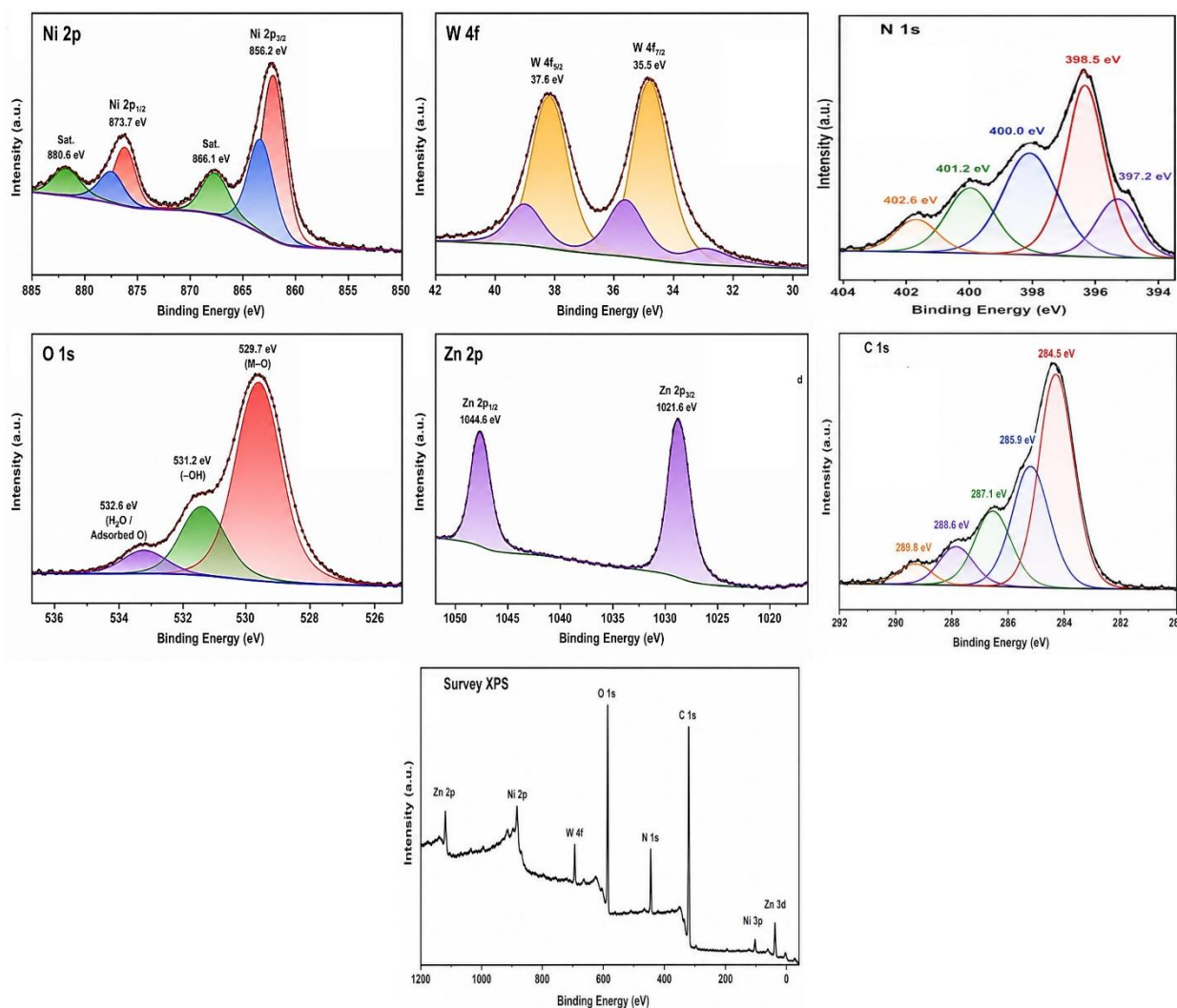
### 3.1.6. X-ray Photoelectron Spectroscopy (XPS) Analysis

The X-ray photoelectron spectroscopy (XPS) analysis also gave compelling evidence for the successful incorporation and chemical interaction of MWCNT, NiWO<sub>4</sub> and ZIF-8 in the hybrid composite wherein the characteristic signals of Zn, Ni, W, O, N and C were distinctly visible in the survey spectrum in Figure 6. The high resolution Ni 2p spectrum shows two major spin-orbit components at 856.2 eV (Ni 2p<sub>3/2</sub>) and 873.7 eV (Ni 2p<sub>1/2</sub>), with satellite features at 864.1 eV and 880.6 eV, respectively. The 2p peak positions and the characteristic shake up satellites are in good agreement with the presence of Ni<sup>2+</sup> (3d<sup>8</sup>) species in NiWO<sub>4</sub>, indicating that the Ni species in NiWO<sub>4</sub> are divalent and not metallic Ni. For the W 4f spectrum, there are two well-defined doublet peaks at 35.5 eV (4f<sub>7/2</sub>) and 37.5 eV (4f<sub>5/2</sub>), with a spin-orbit separation of ~ 2.0 eV, which is typical for W<sup>6+</sup> in a tungstate environment[29,34]. The lack of a lower binding energy W component also suggests that tungsten is mainly found in the form of W<sup>6+</sup>, not reduced W<sup>5+</sup>/W<sup>4+</sup> species. The O 1s can be split into three contributions at 529.7 eV, 531.2 eV and 532.6 eV. The low-binding-energy peak at 529.7 eV is assigned to lattice

oxygen, which is involved in metal–oxygen bonds, mainly in Ni–O/W–O, while the peak at 531.2 eV is mainly attributed to surface hydroxyl groups and/or defect-associated oxygen[28]. The contribution at 532.6 eV is higher binding energy and is assigned to the presence of adsorbed oxygen-containing species and surface H<sub>2</sub>O, which suggests the chemically active and hydroxylated nature of the composite surface. The Zn 2p spectrum exhibits two strong peaks at 1021.6 eV (Zn 2p<sub>3/2</sub>) and 1045.8 eV (Zn 2p<sub>1/2</sub>), separated by ~24.2 eV, indicating the presence of Zn<sup>2+</sup> present in the framework of ZIF-8[24,35,36]. The N 1s spectrum is composed of components at 397.2, 398.5, 400.0, 401.2 and 402.6 eV. The low binding energy value of 397.2 eV can be attributed to the presence of the Zn–N coordination, whereas that of 398.5 eV corresponds to imine/pyridinic-type nitrogen in imidazolate linker. The C–N component at 400.0 eV can be associated with chemically nonequivalent C–N environments while the higher binding energy peaks at 401.2 and 402.6 eV are assigned to protonated/positively polarized nitrogen and weakly oxidized or surface interacting nitrogen species, respectively. The C 1s spectrum further confirms the presence of the carbonaceous MWCNT component and the organic linker of ZIF-8, as evidenced from the spectra peaks observed at 284.5, 285.9, 287.1, 288.6 and 289.8 eV. The dominant peak at 284.5 eV is a characteristic signal of the graphitic sp<sup>2</sup> C=C/C–C bonding, whereas the higher binding energy peaks represent C–N/C–O, carbonyl and oxygenated carbon environments. The contribution at 289.8 eV may also be for highly oxidized carbon or surface interaction involving  $\pi$ [37]. All of these coupled with the characteristic satellite and spin–orbit features indicate the formation of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid successfully. More importantly, the presence of the metal–oxygen, Zn–N, C–N and graphitic carbon environments suggest that there is close coupling among the three components, which can promote charge redistribution at the interfaces, enhance the electronic conductivity of the MWCNT network, and create chemically active sites on the MWCNT surfaces. This functional hybridization of electronic and structural integration

is very advantageous for the function of the hybrid material, especially when improved charge transport, surface reactivity and/or interfacial stability is needed.

**Figure 6.** XPS spectra of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite: (a) Ni 2p, (b) W 4f, (c) N 1s, (d) O 1s, (e) Zn 2p, (f) C 1s and (g) survey spectrum.



### 3.2. Antibacterial Activity

The antibacterial activity of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid nanocomposite against *Staphylococcus aureus* and *Enterococcus faecalis* was evaluated using the agar well diffusion method (AWD) and the results are shown in Figure 7. The antibacterial activity was determined at different sample volumes 50, 75 and 100  $\mu$ L and compared with the positive and negative

controls. The positive control resulted in an inhibition zone of 15 mm and the negative control an inhibition zone of 10 mm for *S. aureus*. In the case of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid, the inhibition zones were 10, 11 and 13 mm at 50, 75 and 100  $\mu$ L, respectively. The inhibition zones obtained were 10, 11 and 12 mm for *E. faecalis*, and 20 mm and 10 mm for the positive and negative controls, respectively. The clear regions visible around the sample wells in both the agar plates offer a direct proof of the antibacterial activity of the hybrid against the tested Gram-positive bacteria. The progressive increase in the diameter of the inhibition zone with increased sample volume is significant, especially as it shows an antimicrobial response in a concentration-dependent manner. In the case of *S. aureus*, the inhibition zone was observed to be 10 mm at 50  $\mu$ L, 11 mm at 75  $\mu$ L and 13 mm at 100  $\mu$ L. The same behavior was noted with *E. faecalis* and the inhibition zone was 10, 11 and 12 mm depending on the amount of the sample. The activity of the hybrid has been clearly higher at the higher concentrations and lower than the positive controls, but the zone of inhibition produced by the nanocomposite is still lower than the positive controls. This is interesting since the inhibition zone for *S. aureus* is slightly larger at 100  $\mu$ L which indicates that the hybrid may have a slightly higher degree of antibacterial activity toward this organism under the experimental conditions used. The small difference in response of the two bacteria, however, suggests that its activity will be wide spread against both of the tested gram-positive bacteria. The results of the agar diffusion assay should also be analyzed based on the physicochemical properties of the hybrid as inhibition-zone diameter is related to not only intrinsic antibacterial activity but also diffusion of the nanocomposite through the agar matrix. Thus, the observed zones should be interpreted as an indicator of the antibacterial activity rather than as a true measure of the MIC.

The synergistic effect of the three integrated components of MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid can be used to rationalize its antibacterial action. The MWCNT network offers a carbon network with a high surface area and conductivity able to allow close contact between the nanocomposite and bacterial cell envelopes. The long dimension of MWCNTs can enhance the

surface contact with the bacterial cell and can also have a physical perturbation effect on the cell membrane. Moreover, electron transfer between the various components of the hybrid is promoted by the conductive carbon network, which helps improve electron transfer at the interfaces. The  $\text{NiWO}_4$  has metal–oxygen sites, which may be involved in surface-mediated redox reactions, and contain  $\text{Ni}^{2+}$  and  $\text{W}^{6+}$  centers. Such redox activity can lead to production of reactive oxygen species (ROS) including highly reactive oxygen-containing intermediates in the presence of appropriate environmental conditions, which can result in oxidative damage to cellular components. The ZIF-8 component is composed of  $\text{Zn}^{2+}$  centers linked with nitrogen containing imidazolate groups, which provides for a porous structure and a high number of surface sites that are accessible. The porous framework can enhance the effective interaction surface area between the nanocomposite and bacterial cells, and it could aid the adhesion of the microorganisms or cell components on the nanocomposite surface. Zn-containing sites can also interact with biomolecules and bacterial membranes, which could help to disrupt the membrane integrity and cellular metabolism. Several processes can therefore occur simultaneously after the nanocomposite has reached the bacterial surface, such as adsorption, perturbation of the membrane, and interaction of ions with the surface, electron transfer and oxidative stress. These effects can result in increased permeability, intracellular leakage, interference with proteins and enzymes, and loss of viability for bacteria. The presence of MWCNTs is especially important as it can create an efficient interface between  $\text{NiWO}_4$  and ZIF-8 which can lower charge-transfer resistance and enhance the efficiency of the surface-active sites. The intimate combination of  $\text{NiWO}_4$  and ZIF-8 can also form heterogeneous interface containing metal–oxygen and Zn–N environments, providing an electronically conductive scaffold. Interfacial coupling could enhance the number and reactivity of the active site(s) relative to the individual components. However, it is important to note that the generation of ROS, membrane damage and release of metal-ions were not directly measured in the agar diffusion experiment, and thus these should be considered as possible pathways

rather than as proven ones. Complementary experiments like intracellular ROS assays, membrane permeability measurement, electron microscopy of treated bacteria, and live/dead staining and quantitative minimum inhibitory concentration studies would be required to confirm the current findings.

The importance of the antimicrobial properties is that it is proven that the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid has multifunctional structural, electronic and surface properties and its antibacterial activity was measured. The larger the volume of sample, the larger the inhibition zone, thus demonstrating that the more active material that is available to interact with the bacteria, the bigger the inhibition zone. The hybrid shows good activity against both *S. aureus* (maximum inhibition zone 13 mm at 100  $\mu$ L) and *E. faecalis* (maximum inhibition zone 12 mm at 100  $\mu$ L), two Gram-positive species of clinical relevance and with different physiological characteristics. This outcome is of scientific value because it demonstrates that the antimicrobial properties are not limited to one bacterial species. *S. aureus* is a typical Gram positive pathogen that is commonly involved in infections of the skin, wounds, and devices, while *E. faecalis* is an important opportunistic bacterium that is often involved in persistent infections and biofilm formation[35,38]. Activity against both organisms thus points to the potential of the hybrid as multi functional antimicrobial material. The moderate response at 100  $\mu$ L also indicates the need for good contact between the nanocomposite and bacterial cells for maximum inhibition. Overall, the combination of MWCNTs, NiWO<sub>4</sub>, and ZIF-8 represents a rational materials-design approach that combines conductivity, porosity, metal-active sites, and surface interactions all in one. The MWCNT component can serve as electrically conductive pathways and surface contact, NiWO<sub>4</sub> component can be used for chemically active metal-O sites, and ZIF-8 component can give porous structure and Zn<sup>2+</sup> based antibacterial activities. This combination could offer benefits to the single component materials as it creates multiple pathways for bacterial inhibition. The present agar diffusion results therefore indicate that there is potential for use of the hybrid in the suppression of bacterial growth in

antimicrobial coatings, protective surfaces and other functional materials. The results of the present work, however, should be considered as proof of antibacterial activity and not as proof of bactericidal activity since the agar diffusion method is not capable of separating growth inhibition from bacterial killing[39,40]. Mechanistic interpretation and the practical antimicrobial activities of the MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid could be greatly reinforced by additional quantitative analysis such as MIC/MBC, time-dependent bacterial viability, ROS, membrane-damage assays, and biofilm inhibition.

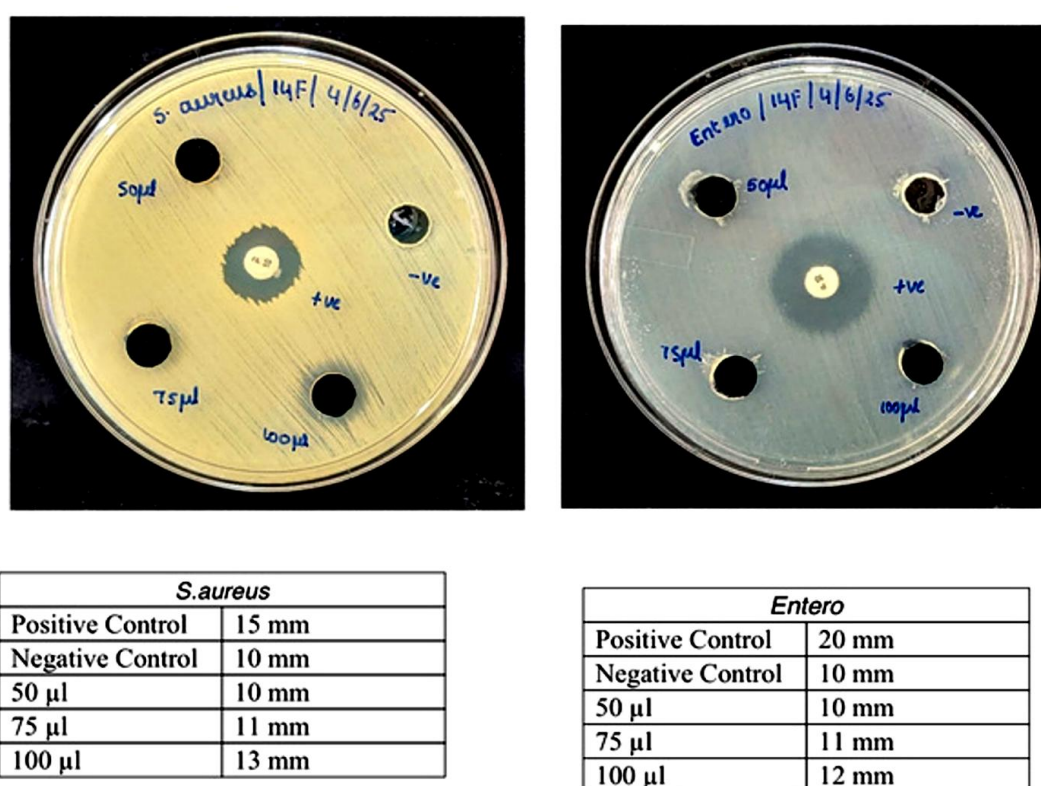


**Figure 7.** Anti-bacterial activity of MWCNT/NiWO<sub>4</sub>/ZIF-8 hybrid nanocomposite against *Staphylococcus aureus* and *Enterococcus faecalis* (Zone of inhibitions).

### 3.3. Antioxidant Activity

The DPPH radical-scavenging activity of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite is shown in Figure. 8. The nanocomposite exhibited a clear concentration-dependent increase in DPPH radical-scavenging activity. The scavenging activity increased from  $15.24 \pm 4.26\%$  at 25 µg/mL

to  $26.57 \pm 4.14\%$  at  $50 \mu\text{g/mL}$  and  $50.77 \pm 4.57\%$  at  $75 \mu\text{g/mL}$ . A further increase was observed at  $100 \mu\text{g/mL}$  ( $74.67 \pm 2.62\%$ ), followed by  $90.00 \pm 2.00\%$  at  $125 \mu\text{g/mL}$ , with the maximum activity of  $96.75 \pm 1.95\%$  at  $150 \mu\text{g/mL}$ . The relatively small standard deviations at the higher concentrations indicate good consistency among the experimental replicates. The progressive enhancement in DPPH radical-scavenging activity with increasing nanocomposite concentration demonstrates a strong concentration-dependent radical-quenching response under the experimental conditions. The enhanced DPPH response may be related to the surface chemical characteristics of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite and the combined contribution of its constituent phases. The MWCNT component provides a graphitic carbon surface that can participate in electron-transfer interactions, while the inorganic NiWO<sub>4</sub> and porous ZIF-8 components may contribute additional surface-active sites. The interconnected multiphase structure may also provide a larger accessible surface for interaction with DPPH radicals. Nevertheless, these proposed contributions should be considered as possible explanations because the DPPH assay alone cannot establish the specific molecular mechanism responsible for the observed activity.

The pronounced scavenging activity at  $125$  and  $150 \mu\text{g/mL}$  ( $90.00 \pm 2.00\%$  and  $96.75 \pm 1.95\%$ , respectively) indicates that the nanocomposite has substantial radical-scavenging capacity at higher concentrations. The antioxidant response therefore complements the antibacterial and cellular biological evaluations of the material and supports its potential for further investigation in applications where radical-scavenging properties are desirable. However, the DPPH assay is an in-vitro chemical antioxidant assay and should not be interpreted as direct evidence of intracellular antioxidant activity. Further cellular oxidative-stress assays would be required to establish intracellular antioxidant effects.

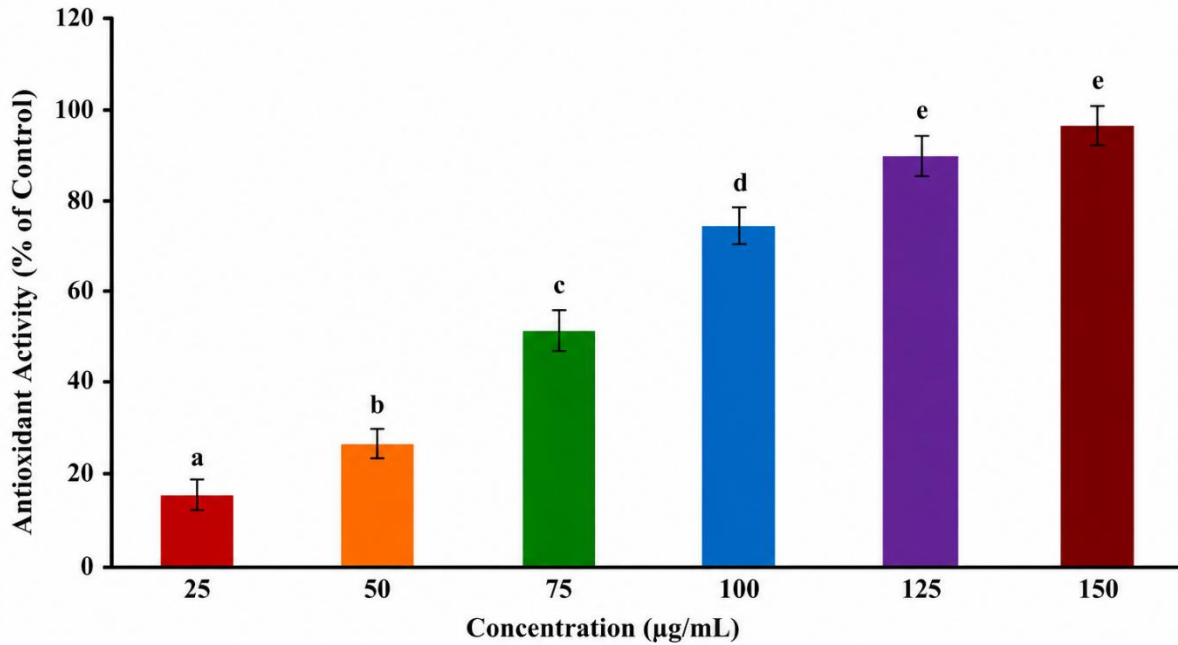


**Figure 8.** DPPH radical-scavenging activity of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite at different concentrations (25–150 µg/mL). Values are expressed as mean ± SD (n = 3). Different lowercase letters above the bars indicate statistically significant differences among concentrations ( $p < 0.05$ , one-way ANOVA followed by Tukey's multiple-comparison test). One-way ANOVA:  $F(5,12) = 287.94$ ,  $p < 0.0001$ .

### 3.4. Cell Viability and Cytocompatibility

The cytocompatibility of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was tested in RAW 264.7 macrophages by means of the MTT assay over a concentration range from 25 to 150 µg/mL. As illustrated in Figure 9, the cells which were not treated had a viability of  $100.0 \pm 2.6\%$ . Treatment with the nanocomposite caused a steady, concentration-dependent reduction in cell viability, the viability dropping to  $92.3 \pm 2.7\%$ ,  $90.4 \pm 2.5\%$ ,  $86.3 \pm 2.1\%$ ,  $81.4 \pm 3.2\%$ ,  $78.1 \pm 2.9\%$  and  $75.4 \pm 3.5\%$  at concentrations of 25, 50, 75, 100, 125 and 150 µg/mL respectively. The decrease in viability as compared to the untreated control therefore varied from about 7.7% at 25 µg/mL to 24.6% at 150 µg/mL. The fact that the standard deviations are relatively small shows that there was good reproducibility among the separate replicates.

The one-way ANOVA revealed a statistically significant effect of treatment concentration on the cellular response measured ( $F(6,14) = 28.36$ ,  $p < 0.0001$ ). The different lowercase letters placed above the bars in Figure 9 show the statistically significant differences between the groups as determined by Tukey's multiple-comparison test ( $p < 0.05$ ). It is important to note that although metabolic activity decreased progressively as the nanocomposite concentration increased,  $75.4 \pm 3.5\%$  of the control response was still retained even at a concentration of  $150 \mu\text{g/mL}$ . These findings show that the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite produced a measurable concentration-dependent effect on the metabolic activity of RAW 264.7 cells, with a considerable amount of metabolic activity remaining throughout the range of concentrations investigated.

The fact that the reduction is concentration-dependent is in agreement with the known behaviour of nanomaterials in cell-based systems, since the cellular response can be strongly influenced by the concentration of the particles as well as by their physicochemical properties, surface chemistry, morphology and the extent of cellular uptake. Specifically, it has already been reported that MWCNTs cause dose-dependent effects in RAW 264.7 macrophages, the cellular responses in these cases being linked to oxidative stress and other kinds of cellular disruption[41]. Nevertheless, the biological response resulting from materials containing MWCNTs is not entirely determined by the mere presence of the MWCNTs; the dimensions of the nanotubes, their aggregation state and their surface characteristics can have a substantial effect on macrophage responses. The response given here therefore illustrates the combined physicochemical properties of the ternary MWCNT/NiWO<sub>4</sub>/ZIF-8 structure since the conductive MWCNT framework offers a carbon interface with high surface area, while NiWO<sub>4</sub> and the porous ZIF-8 add further inorganic and porous surface regions. The different levels of surface accessibility and cellular interaction might affect the metabolic response of macrophages as the concentration of the nanocomposite increases. However, the MTT results

obtained in this study do not by themselves prove a specific mechanism of cytotoxicity or cell death[42].

The MTT assay relies on metabolically active cells reducing a tetrazolium salt and is generally used as a simple colourimetric technique for measuring cellular metabolic activity. Yet when interpreting MTT results for nanomaterials one has to exercise special care since nanoparticles may interfere with colourimetric assays by means of optical absorption, adsorption or by having a direct effect on the assay chemistry. It is therefore correct to interpret the present findings as a concentration-dependent change in cellular metabolic activity rather than as definite proof of cell death. Further evidence about the mechanism causing the observed decrease in MTT response could be obtained using complementary assays such as LDH release, live/dead staining, apoptosis analysis and/or examination of cellular morphology. The MTT results as a whole show that the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite keeps a high degree of cellular metabolic activity in RAW 264.7 macrophages over the entire concentration range tested, with  $75.4 \pm 3.5\%$  of activity remaining at the highest concentration of 150 µg/mL. This result offers a crucial biological background for the later assessment of the nanocomposite's anti-inflammatory activity. Since the anti-inflammatory experiments were also carried out within this same concentration range, the cytokine responses observed should be taken into account together with the relevant cellular viability data and not be interpreted on their own[43].

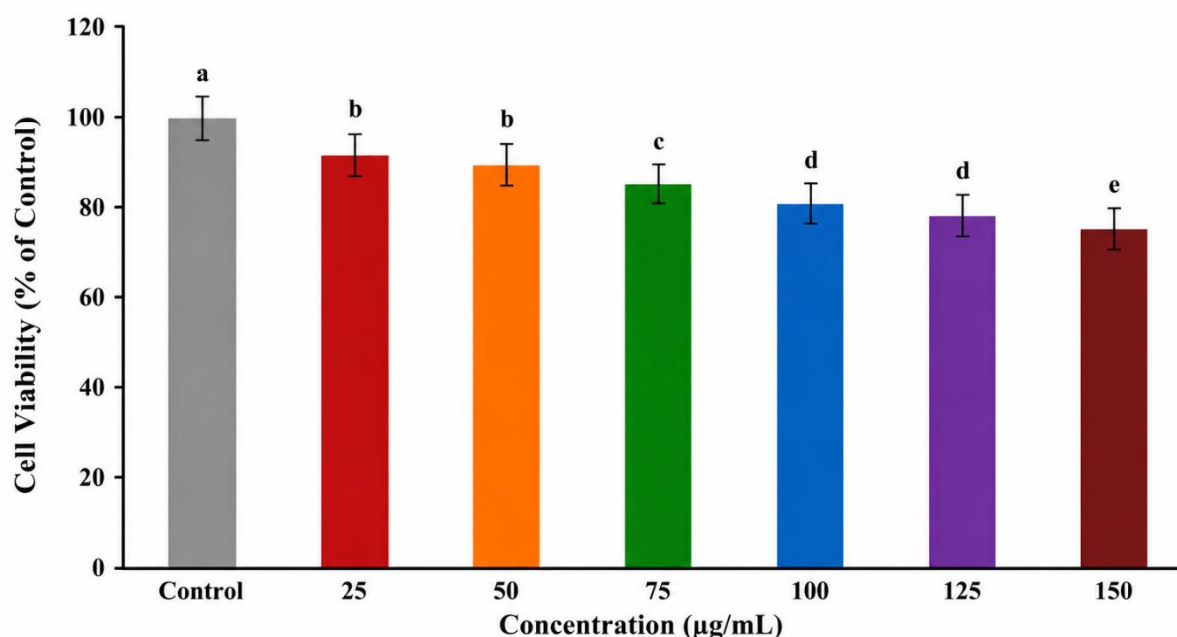


**Figure 9.** Cell viability of RAW 264.7 macrophages treated with MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite at different concentrations (25–150 µg/mL), as determined by the MTT assay. Values are expressed as mean ± SD (n = 3). Different lowercase letters above the bars indicate statistically significant differences among treatment groups ( $p < 0.05$ , one-way ANOVA followed by Tukey's multiple-comparison test). One-way ANOVA:  $F(6,14) = 28.36$ ,  $p < 0.0001$ .

### 3.5. Anti-Inflammatory Activity

#### 3.5.1. Egg-Albumin Denaturation Assay

The anti-inflammatory potential of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite was first assessed using the heat-induced egg-albumin denaturation assay. As shown in Figure. 10, the percentage inhibition of albumin denaturation increased steadily with increasing nanocomposite concentration. The inhibition was 43% at 25 µg/mL and increased to 59%, 71%, 81%, 88%, and 92% at 50, 75, 100, 125, and 150 µg/mL, respectively. Under the same experimental conditions, diclofenac produced inhibition values of 46%, 62%, 74%, 82%, 91%, and 95%, respectively. These findings indicate a clear concentration-dependent protective

effect of the nanocomposite against heat-induced albumin denaturation, with the maximum inhibition of 92% observed at 150  $\mu\text{g/mL}$ . This response was close to that observed for diclofenac (95%) at the same concentration. Similar concentration-dependent inhibition of heat-induced protein denaturation has also been reported for various compounds and materials evaluated using egg-albumin-based in vitro assays[44].

The observed inhibition suggests that the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite has protein-stabilizing activity under the conditions used in this study. The egg-albumin denaturation assay is widely used as an initial in vitro screening method, as the ability to prevent heat-induced protein denaturation can provide preliminary evidence of anti-inflammatory potential. The concentration-dependent response observed in the present study therefore supports the possible anti-inflammatory activity of the nanocomposite. However, this assay is a non-cellular screening model and does not directly demonstrate inhibition of inflammatory signaling or establish a specific molecular mechanism. Therefore, these findings should be considered preliminary evidence of anti-inflammatory potential rather than direct evidence of therapeutic efficacy[45].

At the highest tested concentration of 150  $\mu\text{g/mL}$ , the difference in inhibition between the nanocomposite and diclofenac was relatively small (92% and 95%, respectively), indicating a strong protein-denaturation-inhibitory effect of the nanocomposite. However, the similar inhibition values should not be taken to indicate equivalent pharmacological potency, since the assay reflects protein stabilization under controlled physicochemical conditions rather than the modulation of an inflammatory response in cells. Further studies are therefore necessary to confirm the biological relevance of this activity. In particular, experiments using LPS-stimulated macrophages, along with the measurement of inflammatory mediators such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$ , would provide stronger evidence of anti-inflammatory activity. Additional

mechanistic studies targeting relevant inflammatory signalling pathways would also help clarify the underlying biological mechanisms[46].

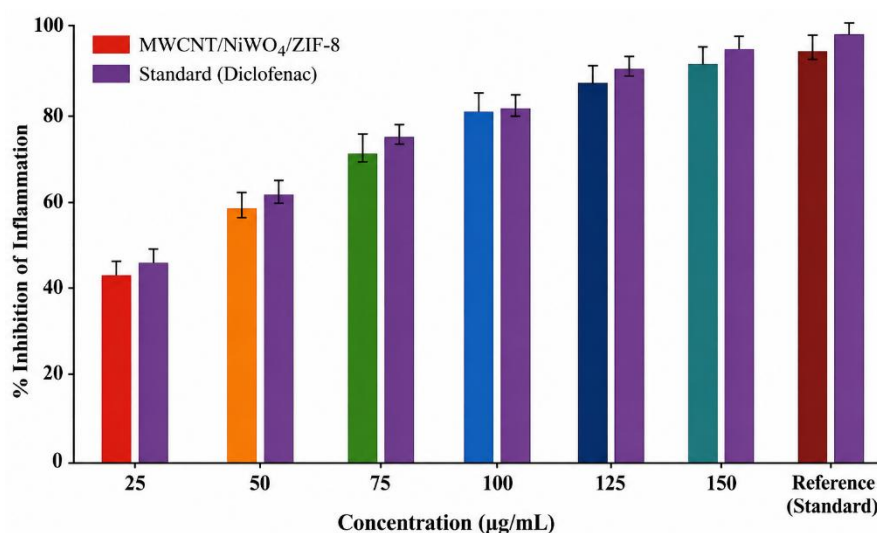


**Figure 10.** In vitro anti-inflammatory activity of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite determined using the egg-albumin denaturation assay at concentrations of 25–150 µg/mL, compared with diclofenac as the reference standard. Values are expressed as mean ± SD of the percentage inhibition of albumin denaturation.

### 3.5.2. Pro-inflammatory Cytokine Modulation

In order to examine more closely the ability of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite to modulate inflammation, the amounts of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were measured in RAW 264.7 macrophages that had been exposed to LPS. As illustrated in Figure. 11, exposure to the nanocomposite caused a concentration-dependent reduction in the relative concentrations of all three cytokines. The level of TNF- $\alpha$  fell from 80% of the level in the untreated control at a dose of 25 µg/mL to 69%, 51%, 37%, 20% and 16% at doses of 50, 75, 100, 125 and 150 µg/mL, respectively. Likewise, the relative concentration of IL-6 decreased from 89% at 25 µg/mL to 75%, 62%, 42%, 26% and 15% at 50, 75, 100, 125 and 150 µg/mL, respectively. For IL-1 $\beta$  as well, a similar pattern was seen, with the level dropping from 90% at 25 µg/mL to 80%, 67%, 52%, 25% and 13% over the same concentration range.

At the highest concentration tested, 150  $\mu\text{g/mL}$ , the relative levels of TNF- $\alpha$ , IL-6 and IL-1 $\beta$  were reduced to 16%, 15% and 13%, respectively, when compared to the level in the untreated control. The fact that all three cytokines showed a consistent decrease as the concentration of the nanocomposite increased shows that there is a strong concentration-dependent modulation of the levels of inflammatory mediators under the experimental conditions[47].

The decrease in TNF- $\alpha$ , IL-6 and IL-1 $\beta$  is in agreement with the anti-inflammatory effect seen in the egg-albumin denaturation assay, in which the inhibition of protein denaturation rose from 43% to 92% within the same concentration range. Taken together, these results offer initial, supportive evidence for the ability of the MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite to modulate inflammation. The observed effect could be due to the nanocomposite's interaction with the cellular processes that are involved in the production of inflammatory mediators; yet the current data do not identify a specific molecular pathway. In particular, it is not possible to definitively assign mechanisms involving NF- $\kappa$ B, MAPK, oxidative stress, COX-2 or iNOS without carrying out direct measurements of these pathways[48].

It is important to interpret the cytokine results together with the MTT cytocompatibility results, which indicated a gradual decrease in metabolic activity from 92.3% at 25  $\mu\text{g/mL}$  to 75.4% at 150  $\mu\text{g/mL}$ . The significant drop in cytokine levels at higher concentrations could therefore be due to a combination of specific modulation of the inflammatory response and changes in cellular metabolic activity or cell number that are associated with concentration. Since the ELISA measurements were carried out on the culture supernatants and the present data have not been normalized to viable cell number or to the total cellular protein, it is not possible from this experiment alone to determine the extent to which the cytokine reduction reflects a direct anti-inflammatory effect. This point is especially relevant when considering the large cytokine reductions seen at 125 and 150  $\mu\text{g/mL}$ [49].

In summary, the coordinated reduction of TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , together with the concentration-dependent inhibition of albumin denaturation, indicates that MWCNT/NiWO<sub>4</sub>/ZIF-8 has the potential to act as a multifunctional material with anti-inflammatory properties. Yet it must be considered that these results constitute only preliminary biological evidence and not proof of a particular anti-inflammatory mechanism or of therapeutic effectiveness. It would be useful to carry out further studies that include normalisation of cell number and cell viability, measurements of intracellular ROS, analysis of COX-2 and iNOS expression, and investigation of the NF- $\kappa$ B/MAPK signalling pathway in order to establish the molecular basis of the observed response[50].

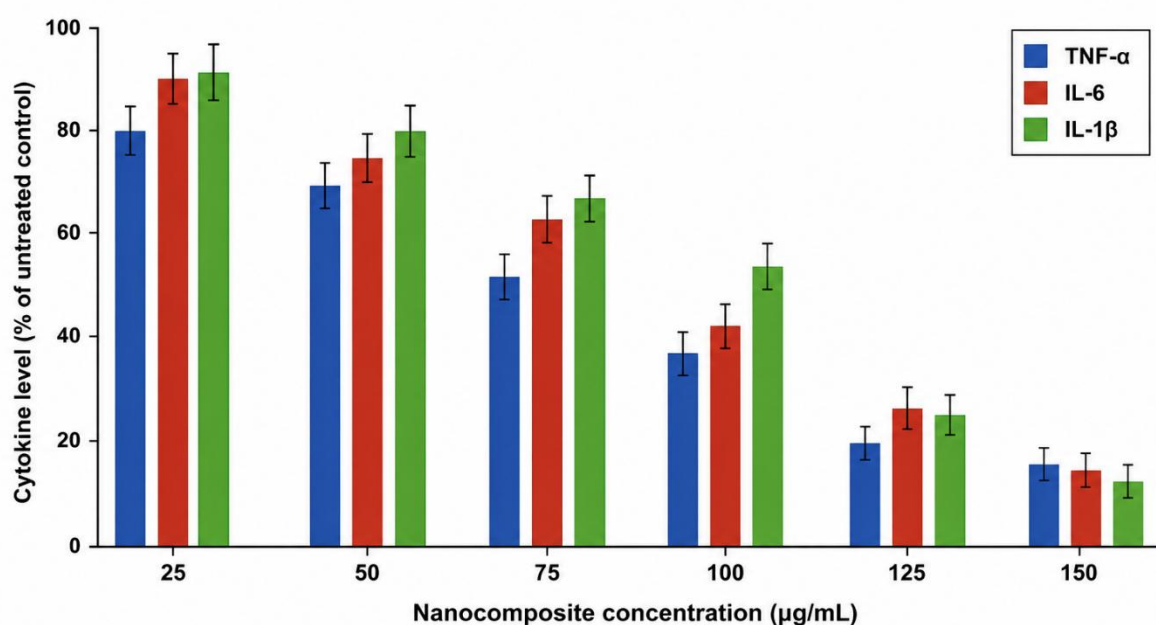


**Figure 11.** Effect of MWCNT/NiWO<sub>4</sub>/ZIF-8 nanocomposite treatment on the levels of pro-inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in LPS-exposed RAW 264.7 macrophages at different nanocomposite concentrations (25–150 µg/mL). Cytokine levels are expressed as a percentage of the untreated control. Values are presented as mean  $\pm$  SD (n = 3)

#### 4. Conclusions

To summarize, the present study has achieved the success in developing a multifunctional material of MWCNT/NiWO<sub>4</sub>/ZIF-8, which has simultaneously high structural stability, high interfacial activity and high biological function within a one-step hybrid structure. The XRD results confirmed the crystalline nature of the material and also the presence of the characteristic structural features of MWCNT, NiWO<sub>4</sub> and ZIF-8, indicating the successful phase integration without any alteration of the basic crystallinity of the combination. The intimate contact and hierarchical organization of the components observed at TEM may allow to obtain an extended interface area, thus providing better interaction with the surrounding environment in an efficient way. Formation of metal–oxygen, Zn–N, C–N and carbon-based structural environments was further substantiated by FTIR and Raman analysis which indicated good chemical compatibility of the constituent phases. The optical response from UV–DRS indicated good electronic interaction in the hybrid structure and XPS analysis confirmed the presence of C, N, O, Zn, Ni, and W and showed that Zn<sup>2+</sup>, Ni<sup>2+</sup>, and W<sup>6+</sup> were the major oxidation states. Most importantly, the integrated architecture encapsulated these physicochemical aspects to translate into significant biological functionality. The nanocomposite has shown an appreciable antibacterial activity with the production of inhibition zones of 13 mm against *S. aureus* and 12 mm against *E. faecalis*. At the same time, its anti-inflammatory response increased progressively from 43% to 150 µg/mL to 92% which was the corresponding standard activity. The combination of the conductive MWCNT framework, chemically active NiWO<sub>4</sub> phase, and the porous Zn<sup>2+</sup>-based ZIF-8 structure resulted in a synergistic effect that provides accessible active sites and favourable interfacial interactions. In conclusion, MWCNT/NiWO<sub>4</sub>/ZIF-8 appears to be a promising advanced multifunctional nanocomposite for antimicrobial and inflammation-modulating applications, warranting further in-depth mechanistic and long-term biological research for practical applications and safety.

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