

Original Research

A Study on Biological Potential of PEG–Chitosan Dually Functionalized Silver Oxide Nanomaterials

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Abstract: This study presented the synthesis of pristine, chitosan, and polyethylene glycol (PEG)–chitosan silver oxide nanoparticles (Ag_3O_4 NPs) using the hydrothermal technique and their potential for biological applications. Nano-systems were characterized via X-ray diffraction (XRD), UV-visible spectroscopy, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy (SEM). The average crystallite size of uncoated Ag_3O_4 NPs was 49 nm, and it is significantly reduced in the case of chitosan and PEG–chitosan conjugation, i.e., 25 nm and 22 nm, respectively. SEM micrographs indicated the spherical morphology of all three nano-systems. The chitosan and PEG–chitosan coating decreases the band gap of Ag_3O_4 NPs. Antibacterial potential was evaluated against *E. coli* and *S. aureus*. The antibacterial potential was enhanced after chitosan and PEG conjugation of Ag_3O_4 NPs. Chitosan-coated Ag_3O_4 NPs were more suited to radical scavenging for antioxidant properties, whereas *in vitro* alpha-amylase inhibition was most significant for PEG–chitosan Ag_3O_4 NPs.

Keywords: silver oxide; polymer functionalization; antibacterial; antioxidant; antidiabetic

1. Introduction

Metal oxide NPs have numerous uses in technology and medicine, and have received a lot of interest as antibacterial agents considering the increased bacterial resistance against prevailing antibiotics [1]. Although several antibiotics are available, resistance to nearly all of them has developed over time [2]. Silver's significant natural antibacterial properties against various pathogens, including bacteria, viruses, fungi, and yeast, are widely recognized [3]. The structural and redox characteristics, surface area-to-volume ratio, adjustable bandgap, exceptional dispersion, remarkable thermal stability, low volatility, and inherent biocompatibility of Ag_3O_4 NPs render them an ideal candidate for diverse biological applications, including wound healing, antibacterial, biofilm inhibition, antidiabetic, antioxidant, and antifungal properties, as well as anti-cancerous actions [4]. Silver oxide nanoparticles (Ag_3O_4) NPs offer added advantages over simple metallic silver NPs owing to the presence of mixed valence states of silver in the silver oxide structure, i.e., ($\text{Ag}^+/\text{Ag}^{2+}/\text{Ag}^{3+}$). This enhances redox action and generation of ROS [5]. In contrast to Ag NPs, which primarily rely on Ag^+ ions, Ag_3O_4 NPs yield sustained oxidative stress using lattice oxygen, consequently improving antimicrobial efficacy. It has been reported that Ag_3O_4 NPs possess superior photocatalytic and biocidal potential as compared to Ag_2O and Ag NPs due to a comparatively narrower band gap and higher surface reactivity [6]. Using steric repulsion, NPs can

be effectively protected against aggregation by covering their surface with layers of neutral organic molecules. By releasing Ag cations in a regulated manner, the distribution of silver NPs in a polymer matrix improves the antibacterial action and may significantly decrease the spread of infectious pathogens [7,8]. Ag cations are evenly distributed throughout the matrix, which inhibits particle aggregation whenever a composition of Ag₃O₄ NPs and a polymer is synthesized [9]. Polymers-coated Ag₃O₄ NPs additionally prevent bacteria from growing and reproducing on their surfaces [2]. Polymers, including PEG and chitosan, are used for coating NPs to extend their biocompatibility, therapeutic effectiveness, and antibacterial activity [10]. Furthermore, polymer films containing metal oxide NPs embedded in them and their surfaces contain antibacterial qualities [1,11].

PEG, a linear synthetic polymer, enhances the colloidal stability and biocompatibility of various metal oxide NPs for biological applications [12]. PEG has a neutral and hydrophilic nature. In addition, PEG coating reduces the harmful effects caused by potentially hazardous NPs. Chitosan, being relatively affordable to obtain from chitin, is the 2nd strongest natural biopolymer [13]. Chitosan-coated Ag₃O₄ NPs can achieve a dual function of an inorganic and organic component of the nanocomposite while being environmentally friendly, biocompatible, and possessing good antibacterial properties [14]. Chitosan-coated Ag NPs can also eliminate gram-negative (G⁻) and gram-positive (G⁺) bacteria and fungi [15]. Laib et al. [16] found strong antibacterial activity of ciprofloxacin-loaded silver NPs with the highest inhibition zone of 34 mm for *B. subtilis* and *K. pneumoniae*. The synergy was observed due to the effects of ciprofloxacin in inhibiting DNA gyrase and ROS production with Ag NPs and membrane damage, which allowed the enhanced action against G⁺ and G⁻ bacteria. Qadeer et al. [17] observed that PEGylated Ag NPs have shown higher antioxidant activity ($128.72 \pm 3.12 \mu\text{g/mL}$) than pristine Ag NPs. Similarly, PEG-capped Ni_xCo_{1-x}Fe₂O₄ nanocomposites presented moderate antimicrobial properties and increased antioxidant/antidiabetic properties with PEG functionalization. PEG-coating increased total phenolic content (15.9 to 19.3 $\mu\text{g GAE/mg}$) and flavonoid content (8.7 to 9.3 $\mu\text{g QE/mg}$), DPPH scavenging (32.2% to 39.1%), and total antioxidant capacity (17.9 to 18.8 $\mu\text{g AAE/mg}$), which was attributed to the carbonyl/ester groups' presence in PEG [18]. Rajkumar et al. [19] studied PEG-coated CuO nanocomposites, which showed good antioxidant, antibacterial, and antidiabetic properties. The nanocomposites were found to have a strong DPPH scavenging ($81.65\% \pm 1.65\%$ at 100 $\mu\text{g/mL}$, IC₅₀ (73.02 $\mu\text{g/mL}$), ABTS ($70.58\% \pm 1.27\%$), and H₂O₂ ($77.82\% \pm 1.25\%$ at 100 $\mu\text{g/mL}$) scavenging power.

A conflict between the system and the generation of free radicals that protects against them is known as oxidative stress. Different types of triggered oxygen that lead to lipid peroxidation and the potential for inflammation are known as reactive oxygen species (ROS) [20]. Increased phagocyte activation and free radical generation in numerous inflammatory diseases lead to changes in membranes, protein denaturation, and vascular permeability. Because of this, anti-inflammatory or antioxidant drugs are required to reduce inflammation and oxidative stress. Several synthetic antioxidants are presently in use and commercially available; however, because of their toxicity and potential health risks, there is a greater need for natural antioxidants [21]. Therefore, research is being done on novel antioxidants that don't have toxic effects. In the current research work, the surface of Ag₃O₄ NPs is dually functionalized with PEG and chitosan polymers using the hydrothermal method. Dual coating synergizes the steric stabilization ability of PEG (due to its hydrophilic and slightly neutral nature) with the cationic bio-adhesion characteristics of chitosan. This combination (PEG-chitosan) boosts colloidal stability, bioavailability, biocompatibility, and better bacterial membrane adsorption of nanomaterials. In the case of single coating, chitosan-coated NPs may

aggregate in biological media, whereas PEG lacks mucosal adhesion at times. Dual coating can improve the stability of Ag_3O_4 NPs by overcoming these limitations. Moreover, chitosan disrupts bacterial membrane and PEG prolongs retention time, thus demonstrating good synergistic antibacterial activity. Additionally, chitosan enables pH-based release of silver ions while PEG minimizes opsonization. These physicochemical reactions signify the dual functionality of Ag_3O_4 NPs with PEG and chitosan [22–24]. The materials were characterized by using XRD, FTIR, SEM, and UV–Vis spectroscopy. The two-fold coated Ag_3O_4 NPs were tested for their antibacterial, antidiabetic, and antioxidant properties.

2. Materials and methods

2.1. Materials

Silver nitrate ($\text{AgNO}_3 \cdot 6\text{H}_2\text{O}$, DAEJUNG Korea), Sodium hydroxide (NaOH, Icon Chemicals, India), PEG-6000 (DAEJUNG Korea), and Chitosan (Icon Chemicals, India) were used to prepare nanomaterials and functionalize them.

2.2. Synthesis of dually functionalized Ag_3O_4

Silver oxide NPs (Ag_3O_4 NPs) were synthesized using the hydrothermal method, where a 1:4 molar ratio of $\text{AgNO}_3 \cdot 6\text{H}_2\text{O}$ to NaOH was used for maintaining alkaline conditions (pH ~10), which facilitates the formation of Ag(III) species [25,26]. The 0.25M (4.25 g) of silver nitrate and 1M (4 g) of NaOH were dissolved separately in 100 mL of distilled water. NaOH (1 M) solution was added drop by drop to the silver nitrate solution. The pH was maintained at ~10 and stirred at 500 rpm for 2 h before hydrothermal treatment. The solution was put into a Teflon-lined autoclave and thermally heated in an oven at 180 °C for 16 h. Later, the precipitates were processed by centrifugation and rinsed with distilled water thrice to eliminate by-products and contaminants. The retrieved precipitates were put for drying in an oven at 60 °C and subsequently homogenized into a powder form. For functionalization, 0.1 g of each chitosan and PEG-6000 was added before incorporating the NaOH solution, and the same steps were followed as discussed for the preparation of pristine Ag_3O_4 .

2.3. Characterization tools

The structural study was performed using XRD with a D8 Advance Bruker instrument. The analysis was performed between the 2-theta range of 20° to 60°. The SEM (NOVA NanoSEM) was utilized for morphological assessment. The Agilent Technologies Cary 630 FTIR instrument was utilized to validate the presence of functional groups. The optical characteristics were investigated using a UV–Vis spectrophotometer (PG Instrument, T80).

2.4. Antibacterial assessment

The nutrient agar (28 g) was mixed thoroughly in distilled water (1000 mL) in a flask. The solution was sterilized at 121 °C for 15 min by autoclaving. The temperature of the flask was checked by placing a thermometer. The temperature was 80 °C in the beginning and reduced to 40 °C after 75 min. The bacterial strain (0.16 mL) was added to the prepared medium. After that, wells were made with the help of a pipette tip, and 0.1 mL sample solutions were loaded in the wells on the growth medium, while the antibiotic, Ciprofloxacin, was also added in the central well as a positive control. The prepared Petri plates were kept in an incubator for 24 h at 37 °C, and clear zones were formed. Conditions: Triplicate wells per sample, 0.1 mL of 1 mg/mL NP dispersion, Positive control (ciprofloxacin, 10 µg/mL), Negative control (sterile water).

2.5. Antioxidant assay

The antioxidant properties were studied on the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay to assess the free radical scavenging capacity of functionalized Ag_3O_4 at the specific concentration of

10 mg/mL. Ascorbic acid (0.1 mg/mL) was used as a positive control to incorporate positive variations in DPPH activity. In 96-well plates, a previously established methodology (Mustafa et al. [27]), 0.01 mL of prepared solution in methanol (Concentrations: 2.12, 4.25, 6.37, 8.5, and 10 mg/mL) was combined with 0.5 mL of 0.2% DPPH solution. After 1 h incubation in the dark, the optical density was measured in the form of absorbance at 517 nm to ascertain the percentage of scavenging capacity of the tested materials using Equation (1).

$$DPPH \text{ Inhibition } (\%) = \frac{Abs^b - Abs^s}{Abs^b} \times 100 \quad (1)$$

where Abs^b and Abs^s represented the absorbance of the blank and sample, respectively.

2.6. Antidiabetic assay

The alpha-amylase inhibition experiment, as specified by Zohra et al. [28] with minor modifications, was implemented to evaluate the potential of Ag_3O_4 NPs as an antidiabetic drug. To produce the reaction mixture, 0.01 mL of test samples (dilution concentration of prepared-NPs ranging from 200 ppm to 800 ppm), 0.015 mL of PBS (pH 6.8), 0.04 mL of starch solution, and 0.025 mL of alpha-amylase enzyme (0.14 U/mL) were added. The concentrations of NPs were adjusted to 200, 400, 600, and 800 ppm in PBS (pH 6.8). Acarbose was used as a positive control. The reaction time was set to 10 min at 37 °C with absorbance at 530 nm. A microtiter plate reader measured absorbance at 530 nm relative to a blank [29]. Equation (2) was employed to determine the % inhibition.

$$\% \text{ Inhibition} = \frac{Abs^{bl} - Abs^{ss}}{Abs^{bl}} \times 100 \quad (2)$$

where Abs^{bl} is the absorbance of the blank solution and Abs^{ss} means the absorbance of the sample.

2.7. Statistical analysis

For the analysis of the results, a complete randomized design was employed for all the experiments. The antibacterial, antioxidant, and antidiabetic experiments were subjected to one-way ANOVA analysis. All the experiments were conducted in triplicate.

3. Results and discussion

3.1. X-ray diffraction

XRD is a crucial tool for analyzing the crystal structure of synthesized materials. It is often used to obtain detailed information on a material's crystalline structure, phase characteristics, lattice parameters, and grain structure [30]. Figure 1 shows the X-ray spectra of Ag_3O_4 , chitosan-coated Ag_3O_4 , and PEG–chitosan dually coated Ag_3O_4 synthesized by using the hydrothermal process. In the case of Ag_3O_4 , the XRD spectrum presents diffraction peaks of Ag_3O_4 located at 2θ of 27.09° , 33.25° , 38.54° , 44.81° , and 54.90° and attributed to (110), (031), (022), (032), and (–202) planes, respectively. All the diffraction peaks correspond to the JCPDS Card # 00-077-1846, which indicates a cubic phase. However, the introduction of chitosan in the reaction disappears the diffraction peak at 27.09° and also transforms the intensity of the remaining peaks. Additionally, peak broadness is increased with chitosan coating, indicating the shift towards an amorphous phase. The crystallite size (D) is calculated by using Debye–Scherrer's formula (Equation (3)) [31].

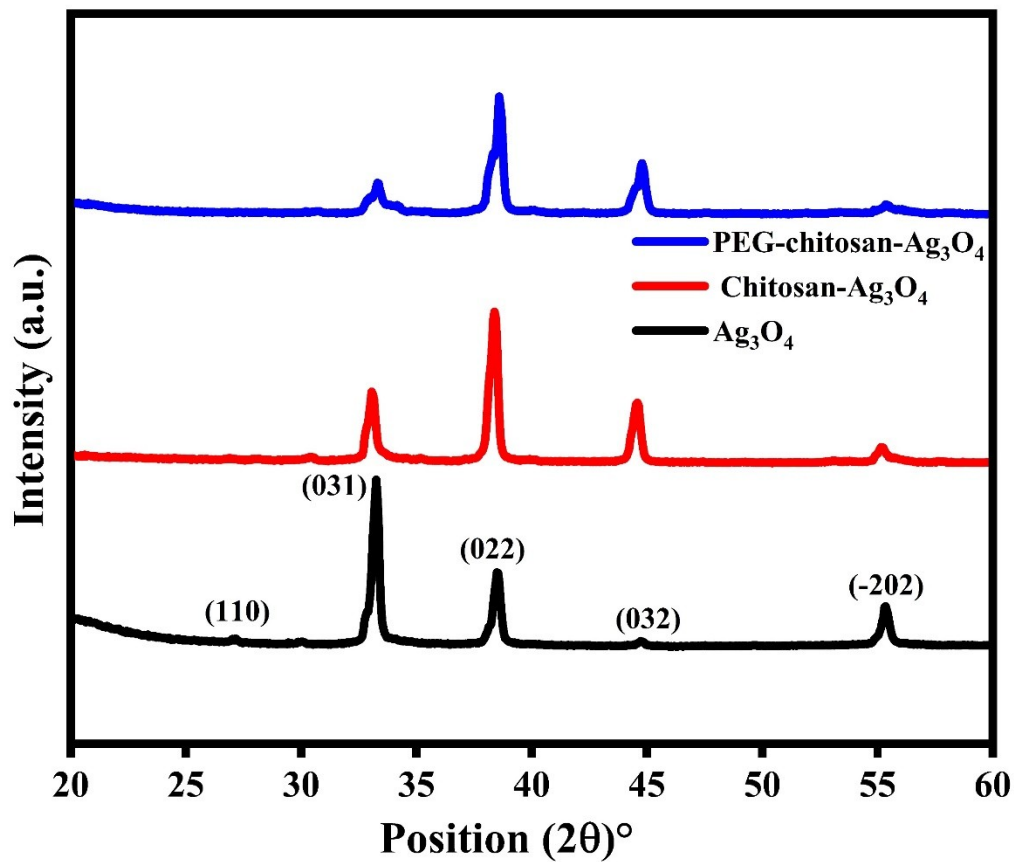


Figure 1. XRD patterns of hydrothermally synthesized Ag_3O_4 , chitosan– Ag_3O_4 , and PEG–chitosan– Ag_3O_4 nanomaterials.

$$D = \frac{K\lambda}{\beta \cos \theta} \quad (3)$$

where $K = 0.9$ is the shape factor, λ represents the wavelength of irradiated X-rays, β is the full width at half maximum, and θ is Bragg's angle. The crystallite size is determined as 49 nm for Ag_3O_4 which decreases to 25 nm (chitosan– Ag_3O_4), and 22 nm (PEG–chitosan Ag_3O_4) (Table 1). A combination of PEG and chitosan reduces the crystallite size of Ag_3O_4 , indicating that polymers limit agglomeration during synthesis [4]. The decrease in size coincides with the bonding of polymer molecules to crystals, which hinders the further development of crystals and influences the morphology, including structure,

size, and size distribution of the nanomaterials [32]. The crystallite size is also calculated through the Williamson–Hall method which shows the same trend as observed in the case of Debye–Scherrer’s formula. However, sizes calculated by the Williamson–Hall method are considerably less than Scherrer’s method, indicating induced defects in the crystals that contract the crystallite size. This explanation is also supported by the calculation of strain and dislocation density which are higher for polymer-coated Ag_3O_4 NPs (Table 1).

Table 1. Structural parameters determined from XRD spectra of Ag_3O_4 , and polymer-coated Ag_3O_4 .

Sample Name	D_{Scherrer}	$D_{\text{W.H}}$	Strain	Dislocation density (lines/m ²)
Ag_3O_4	49 nm	28 nm	0.00025	9.58×10^{14}
Chitosan– Ag_3O_4	25 nm	17 nm	0.00072	1.56×10^{15}
PEG–chitosan Ag_3O_4	22 nm	10 nm	0.00221	2.32×10^{15}

The texture coefficient (TC_{hkl}) is calculated by Equation (4) to determine crystallite growth orientation.

$$\text{Texture coefficient (hkl)} = \frac{\left(\frac{I_{\text{hkl}}}{I_0(\text{hkl})} \right)}{\left(\frac{1}{N} \right) \sum I(\text{hkl}) / I_0(\text{hkl})} \quad (4)$$

The peak with the largest TC_{hkl} indicates the favored growth plane for the formation of crystals. For Ag_3O_4 , the (110) plane has the highest TC_{hkl} value, showing a higher probability of crystal growth in the (110) orientation from the thermodynamic aspect. The polymer-functionalized Ag_3O_4 has a preferential growth direction along the (031) plane, as shown in Table 2.

Table 2. Texture coefficient value for Ag_3O_4 , and polymer-coated Ag_3O_4 .

(hkl)	Ag_3O_4	Chitosan– Ag_3O_4	PEG–chitosan– Ag_3O_4
(110)	4.39	-	-
(031)	0.31	3.82	3.59
(022)	0.02	0.06	0.05
(032)	0.23	0.07	0.03
(-212)	0.04	0.05	0.32

3.2. Scanning electron microscopy

The surface properties and nanoscale morphology of Ag_3O_4 , chitosan– Ag_3O_4 , and PEG–chitosan– Ag_3O_4 nanomaterials were examined using SEM. The low and high magnification SEM images of synthesized nanomaterials are shown in Figure 2(a–f). In the case of Ag_3O_4 , spherical particles are observed with some aggregation and have a particle size of 75 ± 17 nm (Figure 2(a–b)). The chitosan– Ag_3O_4 nanomaterials, as shown in Figures 2(c,d), have spherical particles with a mean size of 35 ± 5 nm. The PEG–chitosan– Ag_3O_4 also presents an identical spherical shape; however, the particle size is considerably larger, i.e., 83 ± 17 nm (Figure 2(e–f)). The observed change in particle

size may be related to the combination structure of Ag_3O_4 and chitosan coating with PEG, which controls the binding of additional atoms to the particles during the synthesis process [33].

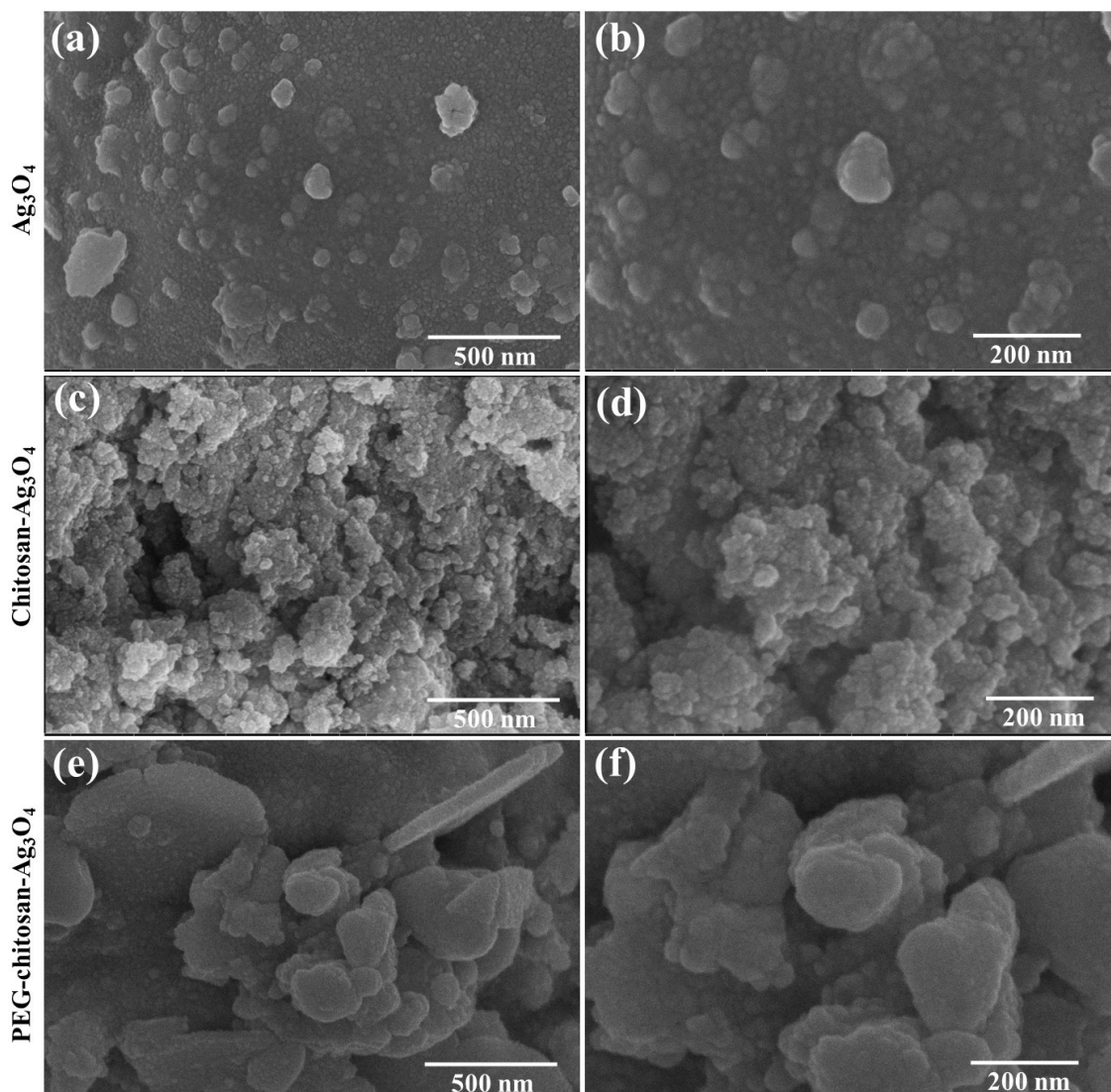


Figure 2. SEM images of (a–b) Ag_3O_4 ; (c–d) chitosan- Ag_3O_4 ; (e–f) PEG-chitosan Ag_3O_4 at low and high magnification.

3.3. UV-vis spectroscopy

UV-vis spectroscopy is used to evaluate the optical properties of Ag_3O_4 -based nanomaterials, as shown in Figure 3(a,b). The absorption curve exhibits typical absorption in the wavelength range of 400–430 nm. Silver is a noble metal, and it undergoes absorption due to its surface plasmon resonance. A surface plasmon represents electromagnetic radiation confined to the surface of a metal and its interactions with the metal's free electrons. The surface plasmon resonance is a specific feature exhibited by metal NPs and cannot be found in the bulk form of the metal [34]. The optical band gap of Ag_3O_4 NPs is found to be 3.48 eV, as shown in Figure 3(b) [35]. But in the case of chitosan- Ag_3O_4 and PEG-chitosan- Ag_3O_4 , the band gap is significantly decreased to 2.3 eV and 2.4 eV, respectively. The presence of chitosan and PEG in Ag_3O_4 may result in extra energy levels. These energy levels can decrease the energy required for electrons to transition from the valence band to the conduction band, thereby narrowing the band gap. This reduction in bandgap is linked with enhanced ROS generation, which ultimately improves the antibacterial properties of the materials [36].

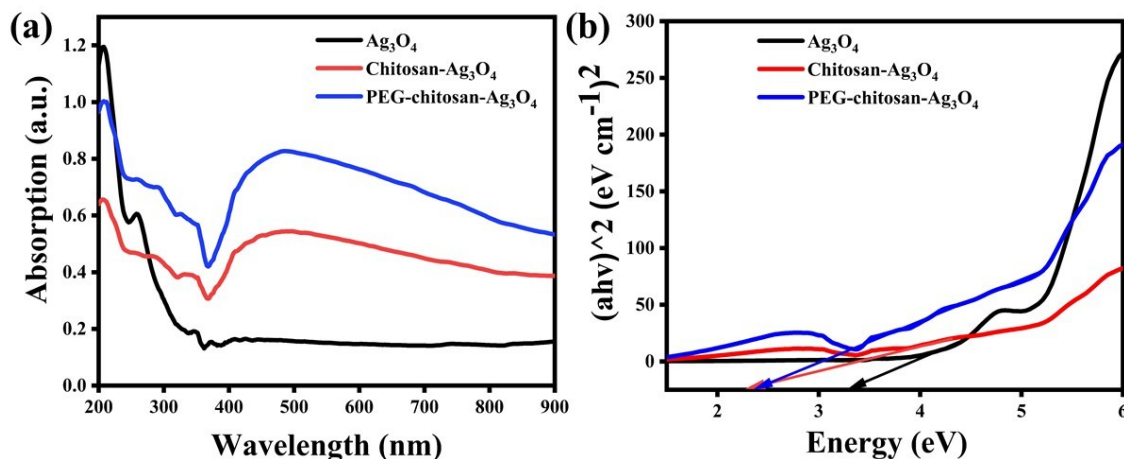


Figure 3. (a) UV-visible absorption curves; (b) Tauc's plot, of Ag_3O_4 , chitosan- Ag_3O_4 , and PEG-chitosan- Ag_3O_4 .

3.4. Fourier transform infrared spectroscopy

FTIR is a useful technique for analyzing the functional groups, vibrational modes, and bond stretching characteristics on the surface of the produced Ag_3O_4 and its combination with chitosan and PEG. FTIR spectra are obtained in the range of $650\text{--}4000\text{ cm}^{-1}$ at room temperature (Figure 4). The band observed at a wave number of 829 cm^{-1} in Ag_3O_4 NPs is associated with the Ag-O bonding [37,38]. The peak at 1523 cm^{-1} in the case of Ag_3O_4 NPs coated with chitosan is due to the N-O stretching. The transmittance band at 1688 cm^{-1} corresponds to chitosan due to C=N stretching [39]. The peaks located at 2000 cm^{-1} and 2112 cm^{-1} are attributed to C-H bending and C=C stretching, respectively. The band at 2325 cm^{-1} belongs to the antisymmetric stretching mode of O=C=O stretching [40]. The transmittance band at 3731 cm^{-1} is related to the stretching of O-H bonds, indicating adsorbed water molecules [41].

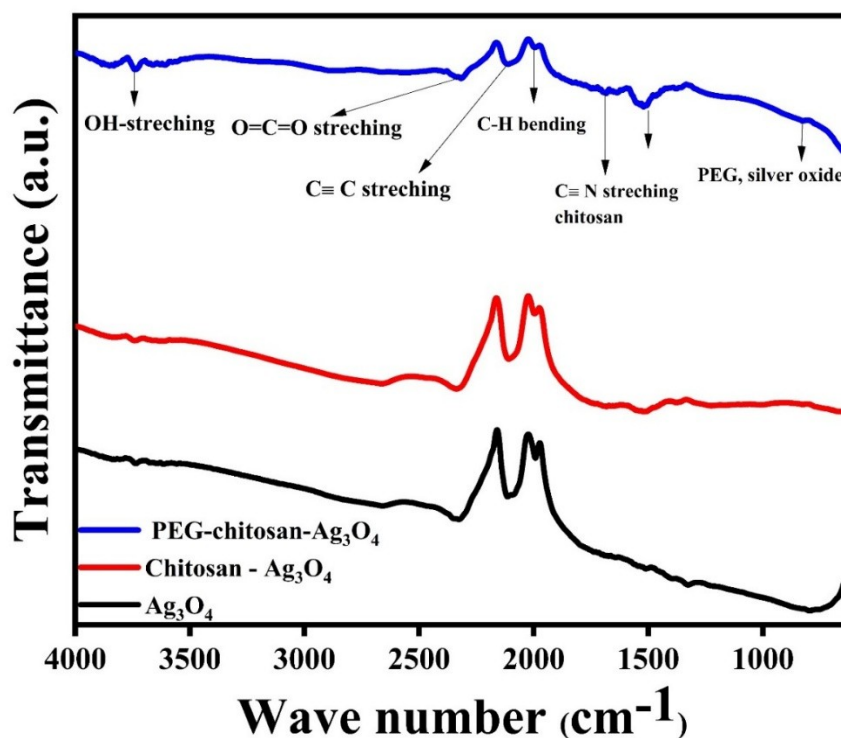


Figure 4. FTIR spectra of Ag_3O_4 , chitosan-coated Ag_3O_4 , and PEG-chitosan- Ag_3O_4 nanomaterials.

3.5. In vitro biological activity

3.5.1. Antibacterial properties

The antibacterial potential of Ag_3O_4 NPs against G⁺ and G⁻ bacterial strains has been well-established over the years. The unique coordination environment of Ag^+ ions and O^{2-} ions in the crystal structure of Ag_3O_4 lays the foundation of the antibacterial properties [42]. Zones of inhibition (in mm) of pristine and chitosan-coated and PEG–chitosan-coated Ag_3O_4 NPs with chitosan and PEG are mentioned in Table 3 and evidenced by Figure 5. The smaller crystallite size (22 nm in the

PEG–chitosan case) increases the surface area, thus enhancing the bacterial contact and ROS generation. Thereby, PEG–chitosan coating has further increased the antibacterial action of Ag_3O_4 NPs. The preferred growth orientation along the (031) plane in the case of polymer functionalized silver oxide NPs may have exposed more active facets for biological interactions. Chitosan coating gives a positive charge to the coated material due to its protonation ability [43]. This increases the strength of NPs' biological interaction with bacterial cells due to electrostatic interaction and ensures uniform dispersion of NPs in the biological medium [44,45]; consequently, the performance of Ag_3O_4 NPs improves. After the attachment to the bacterial cell, Ag_3O_4 NPs generate a significant quantity of ROS [46]. These ROS damage the cell walls of bacteria through oxidative stress [47]. Moreover, silver ions can destroy the electron transport chain; hence, the entire metabolism of the bacterial cell is disturbed [48]. These stress conditions are the major causes behind bacterial inhibition (Figure 6). However, in the case of PEG–chitosan Ag_3O_4 NPs, the antibacterial effect is further improved than chitosan coating. PEG and chitosan both possess intrinsic antibacterial properties [49,50]. After coating with Ag_3O_4 NPs, the antibacterial response is boosted by the synergistic effect of inorganic and organic phases.

Table 3. Zones of inhibition (ZOI) observed for bare and polymer-coated Ag_3O_4 NPs against *S. aureus* and *E. coli*.

Sample	Zone of Inhibition (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
Ag_3O_4	09	10
Chitosan– Ag_3O_4	14	15
PEG–chitosan– Ag_3O_4	15	16
Ciprofloxacin (control)	32	34

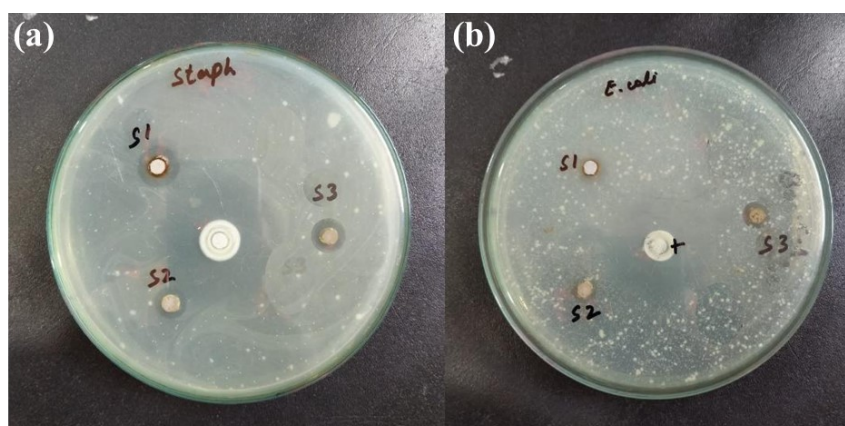


Figure 5. Zone of inhibition for pristine Ag_3O_4 (S1), chitosan- Ag_3O_4 (S2), and PEG-chitosan- Ag_3O_4 (S3) NPs against bacterial strains. (a) *Staphylococcus aureus*; (b) *Escherichia coli*.

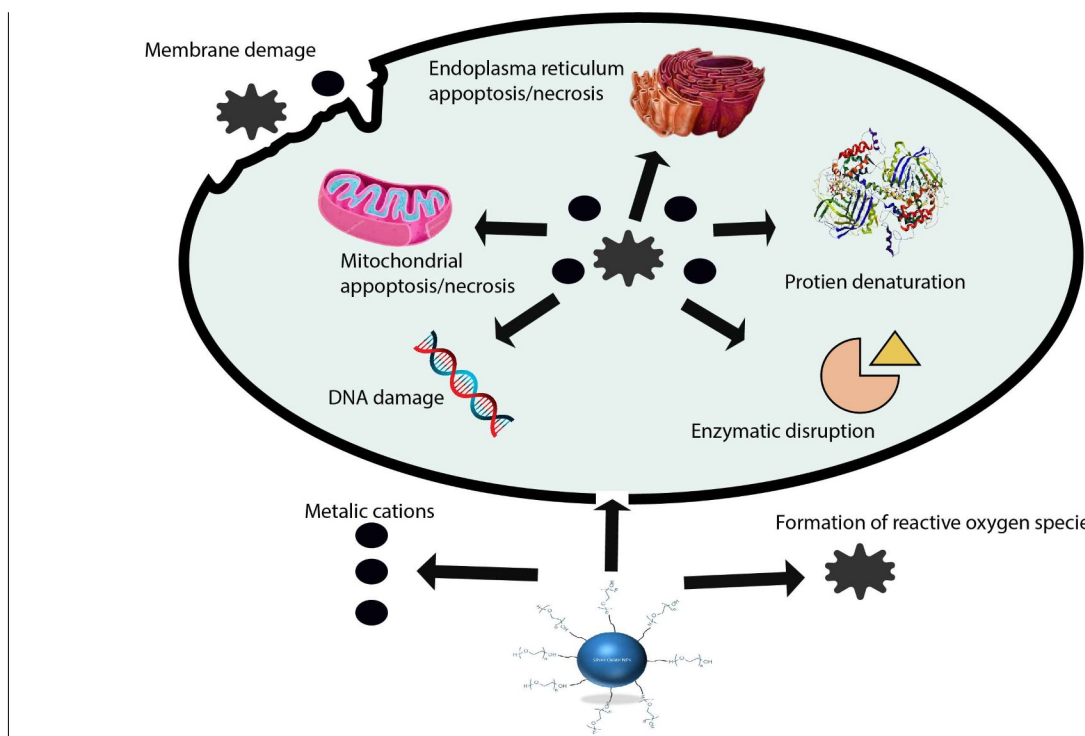


Figure 6. Antibacterial mechanism based on polymer-coated silver oxide NPs.

3.5.2. Antioxidant activity

Antioxidants are chemical substances that neutralize reactive species by inhibiting oxidation processes, thereby preventing cellular damage caused by excessive ROS generation. Natural antioxidants are highly demanded compounds due to their efficacy in regulating ROS-mediated pathogenesis of degenerative disorders, including cardiovascular and carcinogenesis diseases [51,52]. This approach serves to assess the antioxidant capacity in diverse foods, including juices, extracts, vegetables, and biosynthesized nanomaterials. It is a straightforward, cost-effective, and exceptionally sensitive methodology [53]. The DPPH assays are designed to assess the antioxidant activity of biomaterials, i.e., Ag_3O_4 and polymer- Ag_3O_4 . The standard measure for antioxidant activity, referred to as ascorbic acid equivalents, comprises all of these compounds. Figure 7 illustrates that chitosan-coated Ag_3O_4 NPs' antioxidant capacity is significantly enhanced as compared to Ag_3O_4 and PEG-chitosan Ag_3O_4 NPs, with a maximum absorption wavelength at 520 nm. The reduced form of DPPH may donate an oxygen atom, causing the solution to lose its characteristic violet color [54]. The DPPH assay outcomes indicate that chitosan- Ag_3O_4 at 10 $\mu\text{g/mL}$ exhibits the maximum radical scavenging activity of 38%. The lowest radical scavenging activity is measured as 8% for PEG-chitosan Ag_3O_4 . The reduction in antioxidant activity after PEG addition can be attributed to the surface shielding effects and steric hindrance caused by the PEG chains. Cui et al. [55] discussed that the PEG coating might shield chitosan's active sites, resulting in a reduction of radical scavenging. This shows that PEG layer formation on chitosan reduces its antioxidant activity.

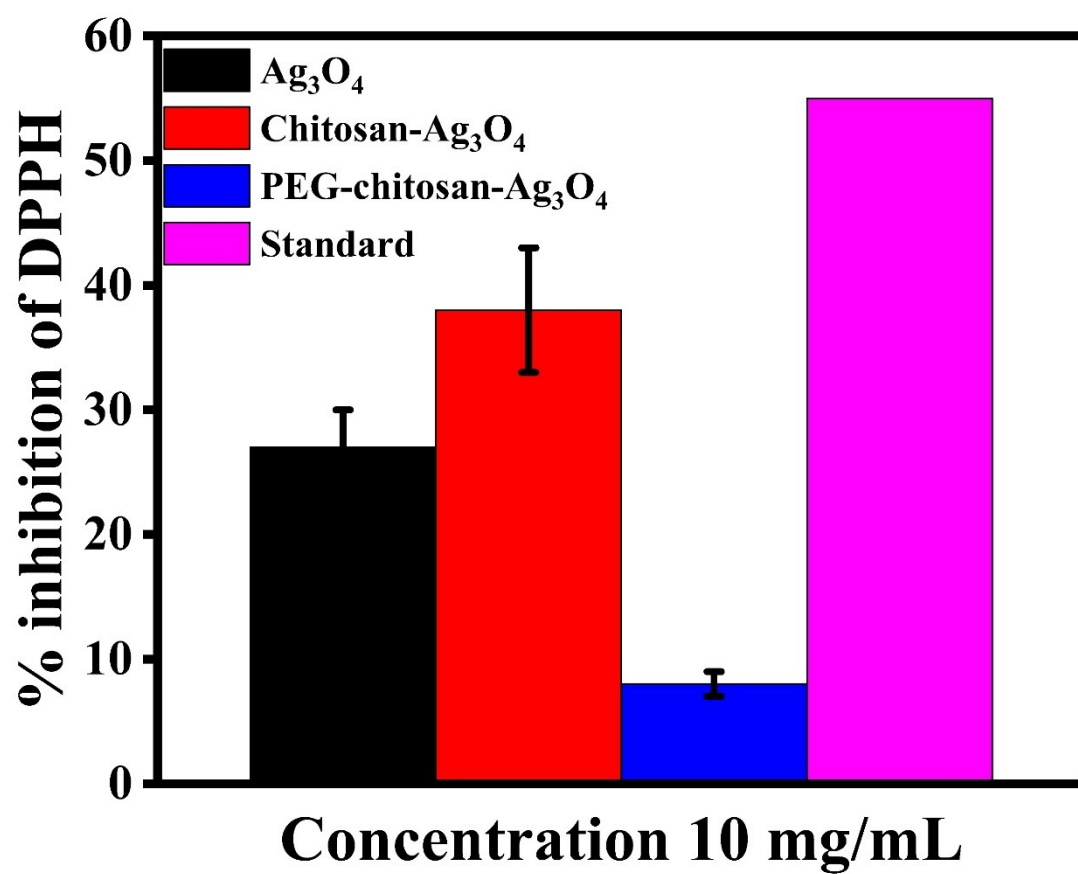


Figure 7. Antioxidant potential (% inhibition of DPPH) of Ag_3O_4 , chitosan-coated Ag_3O_4 , and PEG-chitosan- Ag_3O_4 NPs compared to the inhibition of ascorbic acid.

3.5.3. Antidiabetic analysis

Hyperglycemia occurs due to the insufficient capacity of the body to produce or respond to insulin. Insufficient insulin release in the human body results in diabetes mellitus, which causes the blood glucose level to rise [56]. Diabetes can be maintained artificially by injecting antidiabetic medications to control blood glucose levels. Postprandial hyperglycemia can be prevented by inhibiting enzymes in the intestines responsible for carbohydrate breakdown, with alpha-amylase being a specific target of certain drugs [57]. Various dosages (at dilution ranges from 200 ppm to 800 ppm) of synthesis formulations are employed on *in vitro* assays of alpha-amylase inhibition. The Ag_3O_4 exhibits a dose-dependent inhibitory outcome on alpha-amylase activity. The most significant inhibitory effect of Ag_3O_4 NPs and with polymers on alpha-amylase occurs at a concentration of 800 ppm, whereas the lowest inhibitory effect was noted at a value of 200 ppm (Figure 8). A comparison of materials characteristics and applications is provided in Table 4.

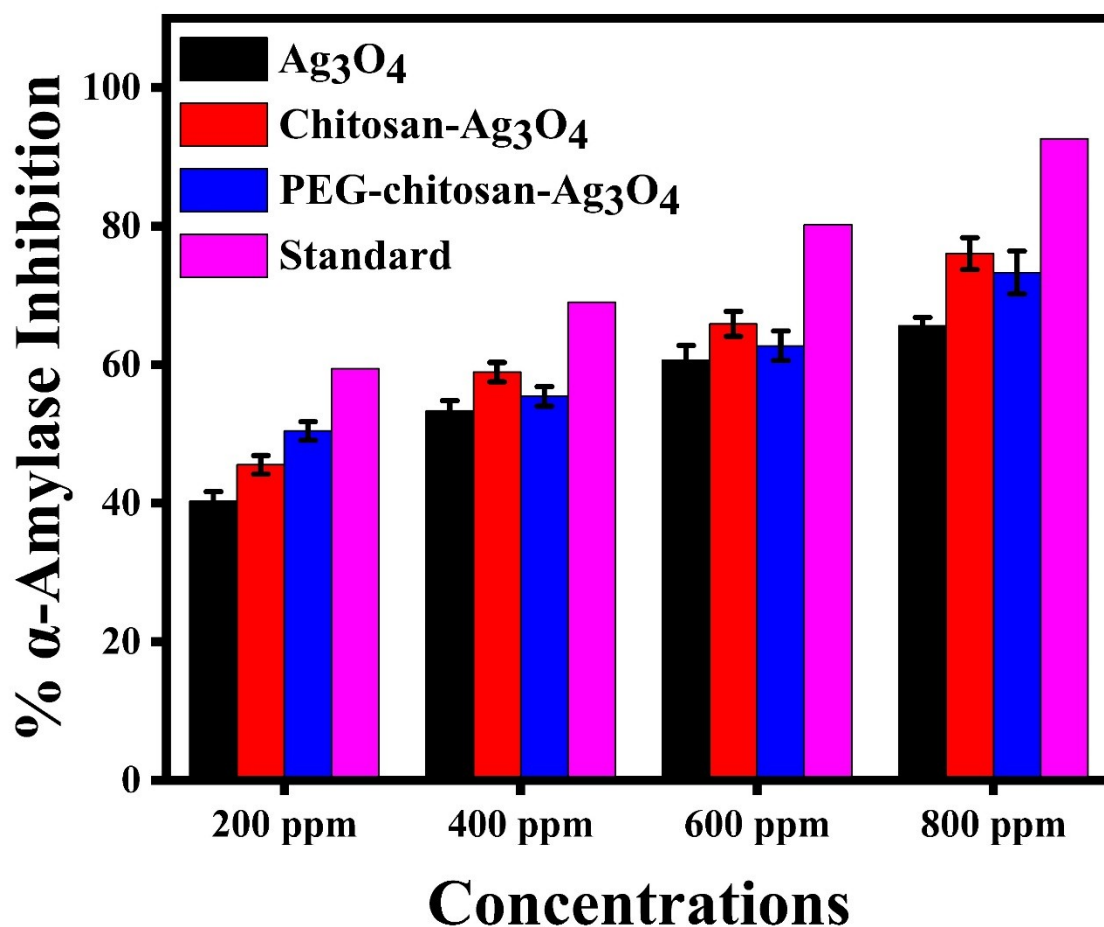


Figure 8. Percentage enzyme (alpha-amylase) inhibition ability of bare Ag_3O_4 , chitosan-coated Ag_3O_4 , and PEG–chitosan-coated Ag_3O_4 nanomaterials.

Table 4. Comparison of structural, optical, and biological properties of pristine, chitosan-coated, and PEG–chitosan-coated Ag₃O₄ nanoparticles.

Parameter	Ag ₃ O ₄ NPs	Chitosan– Ag ₃ O ₄ NPs	PEG–Chitosan– Ag ₃ O ₄ NPs	Significance
Crystallite size (XRD)	49 nm	25 nm	22 nm	Reduced size enhances surface area/reactivity
Bandgap (eV)	3.48	2.30	2.40	Lower bandgap promotes ROS generation
ZOI against <i>S. aureus</i>	9	14	15	Dual coating shows maximal antibacterial synergy
ZOI against <i>E. coli</i>	10	15	16	Dual coating shows maximal antibacterial synergy
DPPH scavenging (%)	28	38	8	Chitosan alone favors antioxidant activity
α -amylase inhibition % (800 ppm)	Good	Best	Best (with minute difference)	PEG–chitosan optimizes antidiabetic potential

4. Limitations

Although the current study presents the improved multifunctional biological efficacy of PEG–chitosan dully coated Ag₃O₄ NPs, certain limitations should be taken into account while interpreting the findings. Firstly, all the biological trials are performed using *in vitro* models, including antibacterial, antioxidant, and enzyme inhibition assays. The results of these assays demonstrate valuable preliminary evidence of biological activity; however, they do not cover the complexity of the *in vivo* physiological conditions. Sometimes, during *in vivo* trials, the behavior of NPs is altered after interacting with biological fluids and causing a change in immune responses and metabolic pathways. Secondly, toxicity and hemocompatibility assays for mammalian cells are not included in this study. Even though PEG and chitosan are generally recognized as biocompatible polymers, detailed biosafety profiling is necessary to support future clinical translation of the PEG–chitosan-coated Ag₃O₄ NPs. Thirdly, the antibacterial potential of the nanomaterials is evaluated by using the agar well diffusion test, which primarily offers diffusion-dependent bacterial inhibition and does not confer quantitative bactericidal information, e.g., MIC, MBC, or biofilm inhibition percentage. Incorporation of such analyses would enhance mechanistic interpretation. Lastly, the long-term colloidal stability and release kinetics of Ag ions in biological media have not been studied, which may affect therapeutic efficacy. These limitations define a clear direction for future investigations and do not detract from the significance of the present study.

5. Conclusion

The present study successfully synthesized dually coated Ag₃O₄ NPs via the hydrothermal method. The dual polymer functionalization reduced the crystallite size to 22 nm and narrowed the optical band gap to 2.3 eV, which enhanced the reactive surface area and electron transfer capability of Ag₃O₄ NPs. The dually coated nanomaterials showed significant antibacterial activity against both Gram-positive and Gram-negative bacteria. Chitosan functionalization demonstrated superior antioxidant potential (38% DPPH scavenging), while PEG–chitosan functionalization exhibited the highest α -amylase inhibition potential of Ag₃O₄ NPs. These findings confirmed the synergistic

integration of PEG and chitosan on the surface of Ag₃O₄ NPs that optimizes the multifunctional biological efficacy, making them potential candidates for advanced biomedical applications in combatting bacterial infections, oxidative stress management, and diabetes-related pathways. The present study was conducted mainly on *in vitro* models. It lacks toxicity profiling, and MIC/MBC data were not observed for *S. aureus* and *E. coli*. This research work can be extended for *in vivo* biocompatibility studies, mechanistic biofilm assays, and pharmacokinetic evaluation of Ag⁺ release.

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