

In Silico and Electrochemical Studies for a ZnO–CuO-Based Immunosensor for Sensitive and Selective Detection of *E. coli*

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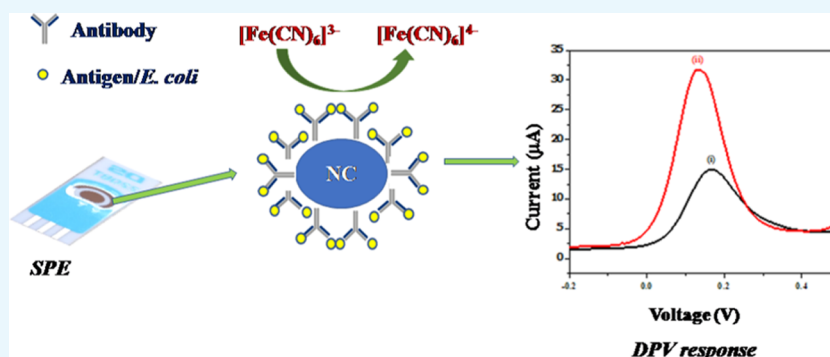


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ABSTRACT: *Escherichia coli* is a harmful Gram-negative bacterium commonly found in the gut of warm-blooded organisms and affects millions of people annually worldwide. In this study, we have synthesized a ZnO–CuO nanocomposite (NC) by a coprecipitation method and characterized the as-synthesized NC using FTIR spectroscopy, XRD, Raman spectroscopy, and FESEM techniques. To fabricate the immunosensor, the ZnO–CuO NC composite was screen-printed on gold-plated electrodes followed by physisorption of the anti-LPS *E. coli* antibody. The biosensor was optimized for higher specificity and sensitivity. The immunosensor exhibited a high sensitivity ($11.04 \mu\text{A CFU mL}^{-1}$) with a low detection limit of 2 CFU mL^{-1} with a redox couple. The improved performance of the immunosensor is attributed to the synergistic effect of the NC and the antipolysaccharide antibody against *E. coli*. The selectivity studies were also carried out with *Staphylococcus aureus* to assess the specificity of the immunosensor. Testing in milk samples was done by spiking the milk samples with different concentrations of *E. coli* to check the potential of this immunosensor. We further checked the affinity between ZnO–CuO NC with *E. coli* LPS and the anti-LPS antibody using molecular docking studies. Atomic charge computation and interaction analyses were performed to support our hypothesis. Our results discern that there is a strong correlation between molecular docking studies and electrochemical characterization. The interaction analysis further displays the strong affinity between the antibody–LPS complex when immobilized with a nanoparticle composite (ZnO–CuO).

1. INTRODUCTION

Harmful pathogenic bacteria cause a variety of diseases in humans and may be naturally present in food and water. *E. coli* causes urinary tract infections, diarrhea, neonatal meningitis, and bacteremia and affects millions of people worldwide.¹ The consumption of contaminated food products primarily causes intestinal infection in which a person suffers from abdominal pain, fever, and diarrhea. Sometimes, it leads to renal failure or severe dehydration. Food safety and public health issues require rapid, specific, and selective detection of pathogens. There are known methods for the detection of bacteria such as the polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), which are slow, expensive, and cannot be used on site.² Thus, there is a need for biosensors that are simple, sensitive, low cost, and can be used by untrained personnel. An impedance-based biosensor for the

detection of *E. coli* (O157:H7) using dose and time response was studied.³ A linear detection range of 2.5×10^4 to $2.5 \times 10^7 \text{ CFU mL}^{-1}$ was reported. Antimicrobial peptides were also exploited in the impedance-based biosensor for the detection of *E. coli*.⁴ Later on, the DNA-based sensor based on surface-enhanced fluorescence was reported for the detection of *E. coli* (O157:H7) using Au@Ag nanorods.⁵ The DNA-based sensor performed in a linear range of 10^{-17} – 10^{-11} M and with a $3.33 \times 10^{-18} \text{ M}$ detection limit. Another research group reported⁶ a

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novel and cheap amperometric immunosensor based on functionalized four-layer magnetic nanoparticles. The linear range of heat-killed *E. coli* (O157:H7) was found from 3.6×10^3 to 3.6×10^6 CFU mL⁻¹. Fang et al. reported an effective way to prepare a reagent-less electrochemical biosensor based on thionine-wrapped *E. coli* and a chitosan-trapped carbon nanodot film modified by a glassy carbon electrode.⁷

In the past two decades, electrochemical-based biosensors have shown great promise in the development of rapid methods for the detection of food-borne pathogens. Immobilization of biomolecules on the transducer surface is considered necessary for the fabrication of biosensors. A recent study reported NC of well-dispersed gold nanoparticles (Au NPs) on the surface of polypyrrole-reduced graphene oxide for the enzyme-free detection of *E. coli* K12.⁸ The enzyme-free sandwiched immunosensor showed excellent analytical performance. The linear range of detection was found to be 1.0×10^1 to 1.0×10^7 CFU mL⁻¹, and the limit of detection was 10 CFU mL⁻¹. Reduced graphene oxide/polyethylenimine functionalized with antifimbrial *E. coli* antibodies was used for selective and sensitive detection of uropathogenic *E. coli* (UPEC) in serum samples using gold electrode (Au–SPE) modifications.⁹ Impedimetric biosensor fabrication was based on using nanomaterials to modify the electrode surfaces to enhance the electrode properties, which received considerable attention.^{10,11}

Au NPs opened new prospects for impedance-based biosensors due to their unique properties as a nanomaterial.^{12,13} Later on, Au NPs deposited on a paper electrode, modified with graphene oxide, for physisorption of a polyclonal antibody were reported for the detection of *E. coli* with an LOD of 150 CFU mL⁻¹.¹⁴ An impedimetric *E. coli* sensor using a biotinylated antibody tethered to a neutravidin self-assembled monolayer (SAM) on a planar gold surface with 10^7 CFU mL⁻¹ of LOD was also reported. An LOD of 1–2 CFU mL⁻¹ using an *E. coli* antibody bound to 11-mercaptopentadecanoic acid SAM on a planar gold electrode and a methoxysilane-modified planar ITO electrode was also reported.^{15–17} An LOD of 10 CFU mL⁻¹ for *E. coli* O157:H7 using a methoxysilane-modified alumina nanoporous membrane for antibody immobilization was reported.¹⁷ A fluorescent magnetic biosensor based on DNazyme was fabricated for the detection of *E. coli* O157:H7. The biosensor indicated a good linear range from 10 to 1000 CFU mL⁻¹. This sensor is feasible for the detection of *E. coli* in drinking water and apple juice.¹⁸ A dual-walker-strategy-based electrochemical sensor for the detection of *E. coli* O157:H7 DNA was also reported. This fabricated sensor exhibits a broad linear range and also allows detection of *E. coli* in real food samples.¹⁹ Recently, silica microspheres were used for the fabrication of a DNA-based impedimetric biosensor for the detection of *E. coli*. The linear range of detection was found to be 1×10^{-10} to 1×10^{-5} μ M.²⁰

Numerous metal oxide nanoparticles have been exploited for developing an efficient electrochemical-based biosensor. ZnO nanoparticles exhibited excellent antibacterial activity due to their photocatalytic activity. Thus, ZnO produced a radical compound that shows antibacterial activity.²¹ CuO nanoparticles are proved to be highly reactive due to their high surface-to-volume ratio, which enhances the antibacterial activity.²² CuO and ZnO combine to form ZnO–CuO NC. The higher the concentration of CuO in composites, the smaller the band-gap energy can be obtained.²³

Herein, we have synthesized and characterized a ZnO–CuO NC via a co-precipitation method. The as-synthesized NC was screen-printed on a Au electrode (SPE) for the detection of *E. coli*. A specific monoclonal antibody was physisorbed on the SPE for electrochemical characterization. The fabricated immunosensor was characterized using CV and DPV. Interference studies were carried out using *S. aureus* to check for the specificity of the immunosensor. Testing in milk samples was also done by spiking the milk samples with different concentrations of *E. coli* to check the potential of this immunosensor.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Materials. Cupric chloride (CuCl₂·2H₂O, 99%), zinc chloride (ZnCl₂, 99%), disodium hydrogen phosphate (Na₂HPO₄, 99%), and sodium hydrogen phosphate (NaHPO₄·2H₂O, 99%) were purchased from Fisher Scientific. The nutrient broth was obtained from HiMedia, ethyl cellulose from CDH, and butylcarbitol acetate (BCA) from Sigma-Aldrich Chemicals Pvt. Ltd. The anti-*E. coli* antibody was obtained from Abcam (ab35654). The structures of zinc oxide and copper oxide were taken from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) while the structures of lipopolysaccharide (LPS) and main receptor protein (PDB Id: 1CFV) of *E. coli* strain O157 were taken from RCSB PDB (<https://www.rcsb.org/>).

The ligand files that were in the .sdf file format were first converted into SMILES format for further analyses using an online web-based converter named Online SMILES converter (<https://cactus.nci.nih.gov/translate/>). Molecular docking analysis was executed using AutoDock Vina.²⁴

2.2. Instruments and Characterization Details. A three-electrode system was obtained from Metrohm, DropSens (DS 220BT). An X-ray diffractometer (Rigaku Ultima IV) was used for phase analysis, Bruker's Tensor 37 spectrometer was used for FTIR measurements, a surface tension/contact angle SEO instrument was used for contact angle measurements, and an IVIUM potentiostat was used for electrochemical characterization. The structural studies were carried out using XRD patterns from 20 to 80° 2 θ scanning range with Cu K α radiation ($\lambda = 1.54184$ Å). An FESEM study at an accelerated voltage of 10 kV was used to find out the shape and surface morphology of ZnO–CuO NC. The FTIR spectrum was recorded in an ATR mode from 4000 to 590 cm⁻¹, and Raman spectroscopy characterization was carried out from 0 to 1000 cm⁻¹ range.

The electrochemical characterization was carried out using CV and DPV. For CV measurements, 60 μ L of a serially diluted sample was purged on SPE to record the spectrum at a 50 mV s⁻¹ scan rate and from –1 to +1 V voltage. The anti-*E. coli* antibody (1:40 dilution) was physically immobilized via the drop-casting method and dried at 4 °C for 1 h. Physisorption/physical absorption (bioconjugation process) of an antibody is the simplest method of antibody immobilization, which does not require additional reagents or linkers. The presence of linkers might lead to cross-reactivity with the antibody and interference with specificity as well and may also affect the functionality of the antibody. The CV measurements were recorded in a solution of a ferri/ferrocyanide redox couple (1:1) in PBS (0.1 M, pH 7) as a background electrolyte at room temperature. Similarly, DPV studies were carried out at potential from –1 to +1 V, pulse time 10 ms, and pulse amplitude 10 mV. For selectivity studies,

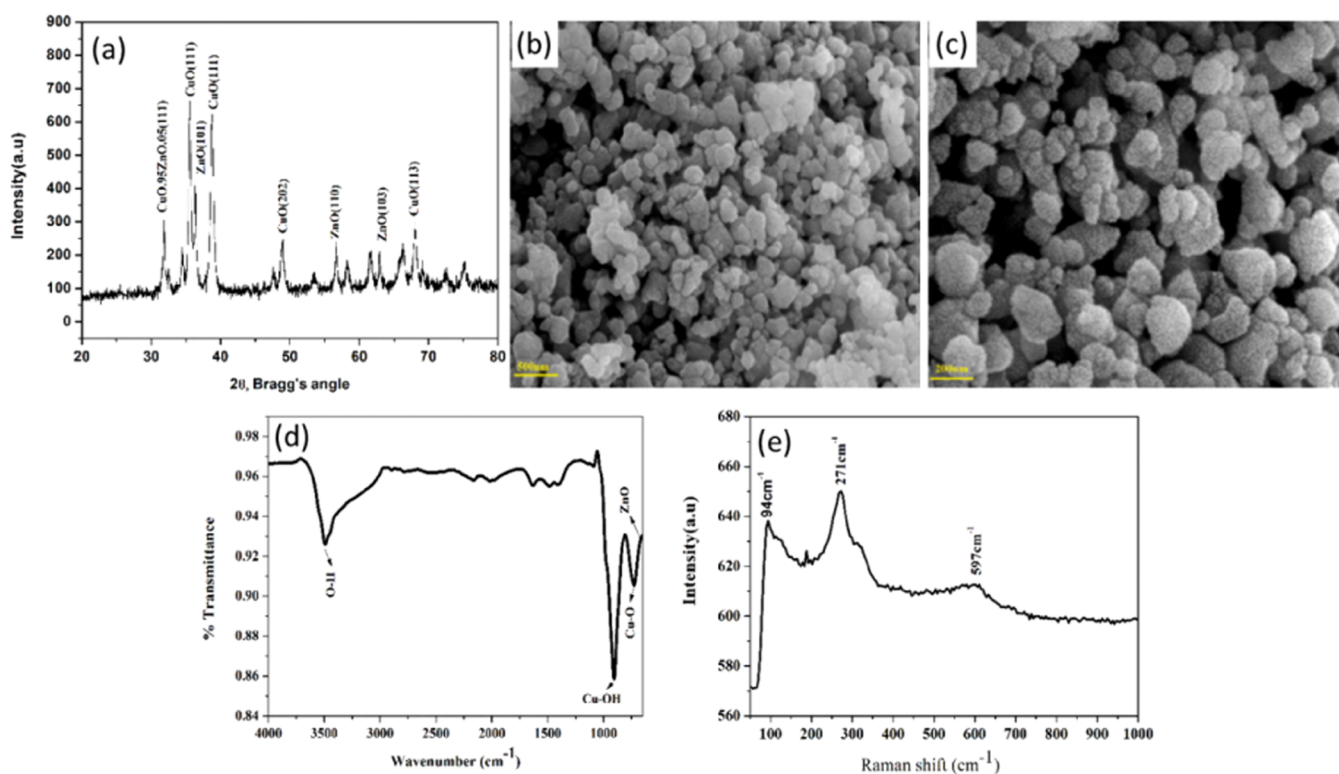


Figure 1. (a) XRD profile for ZnO–CuO NC, (b) FESEM image of ZnO–CuO NC at low resolution, (c) FESEM image of ZnO–CuO NC at high resolution, (d) FTIR spectra of ZnO–CuO NC, and (e) Raman plot for ZnO–CuO NC.

S. aureus was used as an interfering agent. The fabricated immunosensor was also tested in milk samples.

For molecular docking studies, the LPS of strain O157 was selected as the main receptor for the study. The target structures are not used directly for docking as they may contain heavy atoms, co-crystallized ligands, water molecules, metal ions, co-factors, etc. Therefore, they first need to be preprocessed. The preprocessing was done by the addition of hydrogen atoms and addition of polar charges and Gasteiger charges. Once the preprocessing was done, the target receptors were converted and saved in the .pdbqt format. Nano-composites of zinc and copper oxides were selected as the ligand for the molecular docking study. The energy minimization was executed using mmff94 force field, optimization was done using steepest descent (SD) algorithm in a total of 200 steps, and the terminating criteria was updated in case the energy difference was less than 0.1. The ligand was later on converted from the .sdf to the .pdbqt format for docking purposes. Receptor optimization: The 2POF structure was optimized by first removing water molecules, any bound co-factors, metal ions, and co-bounded ligands and later on screened for a post-translational modification (PTM) check using the Vienna PTM tool.²⁵

Docking was executed on AutoDock Vina on a Windows 64-bit platform.²⁴ The same initial procedure of preprocessing was carried out for each target separately. The synthesized ligand was uploaded after the prepared target using predefined parameters. The binding pocket was set to the original ligand referred to all of the atoms within 10 Å distance. The Broyden–Fletcher–Goldfarb–Shanno run (BFGS run) was used as the main optimization algorithm for docking. After docking, atomic charges were computed using the Atomic Charge Calculator II webserver.²⁶ The electronegativity

equalization method (EEM) was used to calculate the atomic charges of the two complexes.²⁷ Further, the interactions formed between the antibody–nanocomposite formulated structures were identified using a protein ligand interaction profiler.²⁸

2.3. Synthesis of ZnO–CuO NC. ZnO–CuO NC was synthesized using $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and ZnCl_2 in distilled water. A solution (0.05 M) of ZnCl_2 was added to 0.1 M solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ with continuous stirring. The mixture solution obtained was then precipitated with an ammonia solution at pH 11. The precipitate was washed with distilled water and dried at 80 °C and finally calcined in a muffle furnace at 600 °C for 4 h.

2.4. Fabrication of the ZnO–CuO-Modified Electrode. For the fabrication of the immunosensor, the SPE (three-electrode system) was first used, which consisted of a working electrode (WE) and a counter electrode (CE) made up of gold and silver as a reference electrode (RE). The WE of 4 mm in diameter was coated with NC using a screen-printing technique. The SPE was dried at 60 °C for 1 h.

2.5. Bacterial Cultivation. The bacterial culture was prepared by inoculating a 10 μL loop of *E. coli* in nutrient broth for about 18–20 h at 37 °C. The culture obtained was centrifuged three times, and the pellet obtained was suspended in phosphate buffer saline (PBS, pH 7.2). The absorbance was recorded at 600 nm to find out the initial cell count using the McFarland standard method (it is the method used to estimate the concentration of different bacteria based on the turbidity of the bacterial suspensions). For this study, this method was used to find out the concentration of bacteria in CFU mL^{-1} ²⁹ and to prepare the serially diluted samples of the bacterial culture (1×10^3 to 1×10^6 CFU mL^{-1}).

3. RESULTS AND DISCUSSION

3.1. Characterization of NC. The results of the XRD analysis (Figure 1a) showed that CuO diffraction patterns

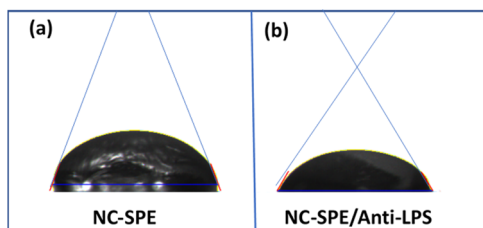


Figure 2. Contact angle images of (a) NC-SPE and (b) NC-SPE/Anti-LPS.

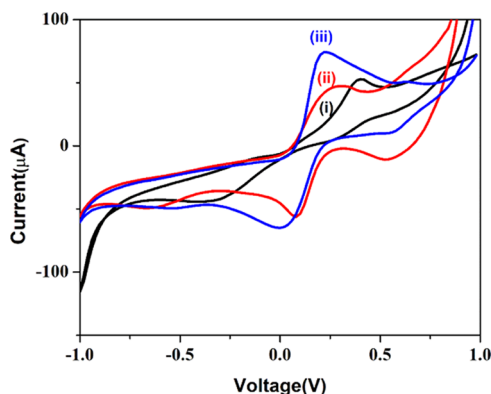


Figure 3. CV response of (i) buffer, (ii) NC-SPE and (iii) NC-SPE/Anti-LPS at fixed *E. coli* concentrations.

appear in the peak 2θ at 35.56, 38.8, 48.46, and 68.03, which match well with the JCPDS card number 895899. The 2θ values of 36.32, 56.64, and 62.96 correspond to ZnO, which also match well with the JCPDS card number 891397. The peak at 31.63 corresponds to CuO_{0.95}ZnO_{0.05}, which matches well with the JCPDS card number 880268 and confirms the formation of ZnO–CuO NC. An FESEM study at an accelerated voltage of 10 kV was used to find out the shape and surface morphology of ZnO–CuO NC, and the corresponding images are shown in Figure 1b (low resolution) and Figure 1c (high resolution). The shape cannot be properly identified due to the agglomeration of the particles, though the particle size obtained was about 25 nm.

The absorption band of each specific chemical bond has unique energy; therefore, FTIR spectroscopy can be employed to obtain structural and bond information of a complex as well as the strength and type of bonding.³⁰ For elemental characterization (Figure 1d) using FTIR, the O–H stretching band was observed at 3491 cm^{−1}, which was due to water used as a solvent during the synthesis of the NC. A peak at 906 cm^{−1} corresponds to Cu–OH. Similarly, the peaks at 723 and 669 cm^{−1} correspond to Cu–O and Zn–O bonds, respectively. Raman spectroscopy is a versatile technique to study vibrational properties. The three major peaks were observed at 94, 271, and 597 cm^{−1} (Figure 1e). The major peak in the Raman spectrum, at 271 cm^{−1}, corresponds to CuO, whereas two other peaks at 94 and 597 cm^{−1} correspond to ZnO.

3.2. Contact Angle Studies. The hydrophilicity and surface wettability of an electrode depend on surface chemistry. Contact angle measurements were carried out for

both NC-SPE (without antibody immobilization) and NC-SPE/Anti-LPS (after antibody immobilization) immunoelectrodes (Figure 2). A buffer droplet (PBS buffer with a ferri/ferro redox couple) was produced over both the electrodes using a micropipette, and the contact angle was measured at the interface of water and the electrode surface. The contact angle for NC-SPE was 70.41° (Figure 2a) and for NC-SPE/Anti-LPS was 61.4° (Figure 2b). Due to the excess availability of –COOH groups in the NC-SPE/Anti-LPS immunoelectrode, the surface hydrophilicity was increased compared to that of NC-SPE. This indicates that antibody immobilization increases the surface hydrophilicity and thus increases the interaction between the antibody and antigen. Apart from this increase, hydrophilicity resulted in stronger adhesion of biomolecules on the surface of the NC.

3.3. Electrochemical Characterization of the Fabricated Immunosensor. Electrochemical characterization was carried out at room temperature. The electrochemical behavior of (ii) NC-SPE and (iii) NC-SPE/Anti-LPS was studied using CV at a scan rate of 50 mV s^{−1} with a voltage scan from −1 to +1 V. All of the studies were carried out in a PBS solution containing K₃[Fe(CN)₆]/K₄[Fe(CN)₆]. A well-defined redox peak was observed for both the electrodes (Figure 3). A drastic increase in anodic current was observed after antibody immobilization (NC-SPE/Anti-LPS). This may be due to formation of the antigen–antibody complex on the surface of the electrode, and because of increased antigen binding sites, the complex behaves as an accelerating layer for the transfer of electrons.³¹ The I_{pa}/I_{pc} ratio for NC-SPE electrode was found to be 0.8 which indicates the quasi-reversible nature of the electrode, whereas the I_{pa}/I_{pc} ratio for NC-SPE/Anti-LPS electrode was found to be 1.16 which shows the reversible nature of the fabricated electrode.

3.4. Effect of Scan Rate and Immunosensor Response Studies. The charge transfer characteristics of the immunosensor were measured using kinetic studies from 10 to 100 mV s^{−1} scan rates on NC-SPE as well as the NC-SPE/Anti-LPS immunoelectrode (Figure 4). The peak current (a, b) of the anode and the cathode increases with scan rate, indicating the continuous and homogenous electron transfer on the electrode.³² The anodic and cathodic peak currents were plotted against the square root of scan rate (c, d), which indicates a linear relation (>30 mV s^{−1}) with R^2 values close to 0.99.

This linearity suggests that the mobility of the charge carriers was a diffusion-controlled process.³³ Variations in redox current with the log of scan rate were also plotted to understand the diffusion-controlled process (Figure 4e,f). The value of the slope, intercept and the linear regression of linear behavior of the peak current were calculated by linear fitting, eqs 1–4.

For the NC-SPE immunoelectrode:

$$I_{pa} = -3.55 \mu A + 7.7 \mu A(mV/s)^{-1/2} \times [\text{scan rate}]^{1/2};$$

$$R^2 = 0.99 \quad (1)$$

$$I_{pc} = 9.28 \mu A - 8.87.7 \mu A(mV/s)^{-1/2} \times [\text{scan rate}]^{1/2};$$

$$R^2 = 0.99 \quad (2)$$

For the NC-SPE/Anti-LPS immunoelectrode:

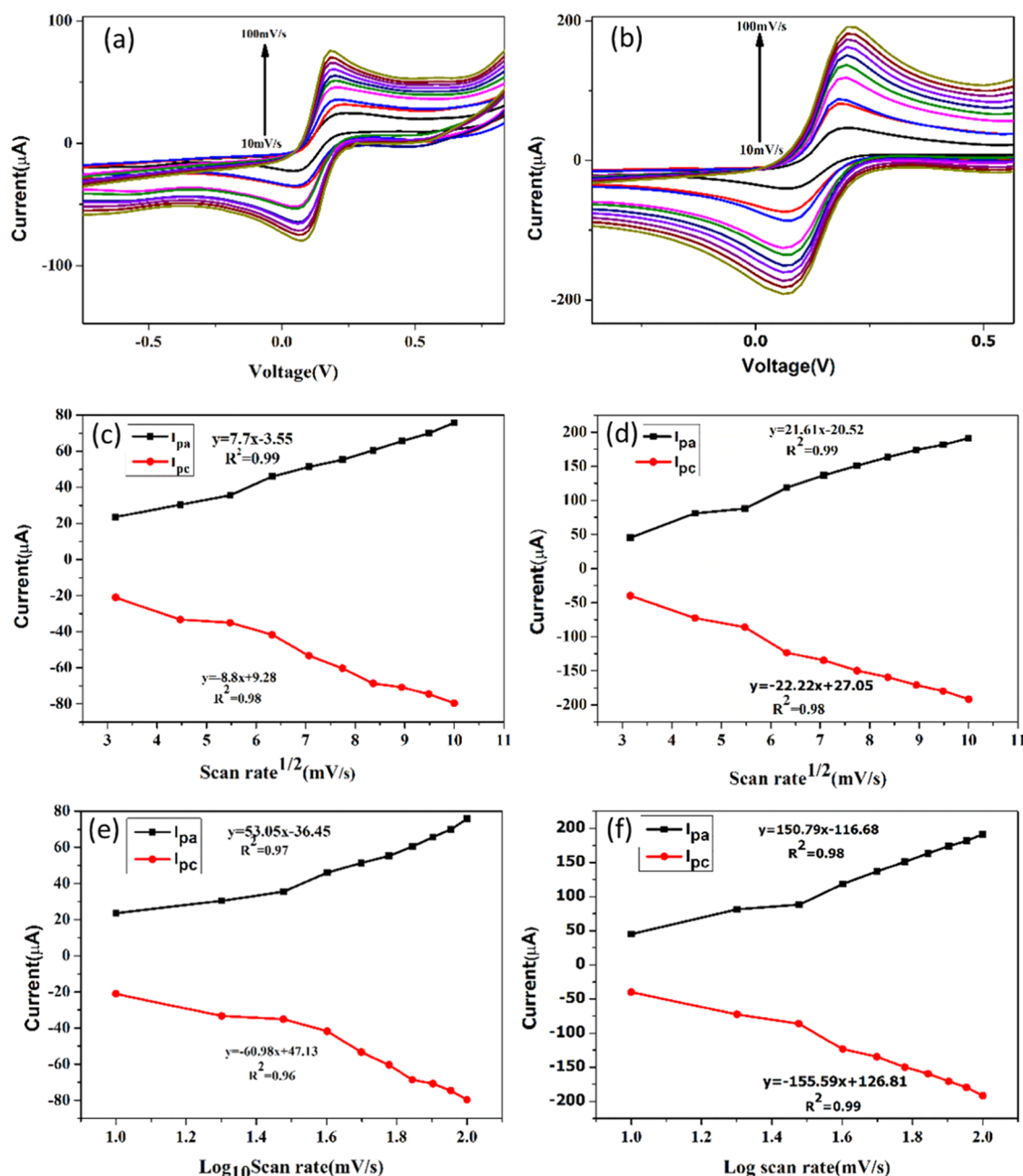


Figure 4. (a, b) Cyclic voltammograms of NC-SPE at fixed concentrations (8×10^4 CFU mL⁻¹) from 10 to 100 mV s⁻¹ scan rate for NC-SPE and NC-SPE/Anti-LPS immunoelectrodes, respectively. (c, d) Variation in redox current with the square root of scan rate for NC-SPE and NC-SPE/Anti-LPS, respectively. (e, f) Variation in redox current with the log of scan rate at fixed concentrations (8×10^4 CFU mL⁻¹) at a scan rate ranging from 10 to 100 mV s⁻¹ for NC-SPE and NC-SPE/Anti-LPS, respectively.

$$I_{pa} = -20.52 \mu A + 21.61 \mu A (mV/s)^{-1/2} \times [\text{scan rate}]^{1/2};$$

$$R^2 = 0.99 \quad (3)$$

$$I_{pc} = 27.05 \mu A - 22.22 \mu A (mV/s)^{-1/2} \times [\text{scan rate}]^{1/2};$$

$$R^2 = 0.98 \quad (4)$$

The diffusion coefficient, effective electrode surface area, and surface concentration of electroactive species for both NC-SPE and NC-SPE/Anti-LPS immunoelectrodes were calculated.

The value of the diffusion coefficient was calculated from the Randles–Sevcik equation³¹

$$D = \frac{I_p^2}{(2.69 \times 10^5)^2 A^2 n^3 C^2 v} \quad (5)$$

D is the diffusion coefficient (cm² s⁻¹), I_p is the maximum peak current (ampere), A is the electrode surface area (0.12 cm²), n is the number of electrons (1), C is the concentration of PBS buffer (0.1 M), and v is the scan rate (V s⁻¹). The diffusion coefficients for NC-SPE and NC-SPE/Anti-LPS were found to be 1.1×10^{-14} and 7.05×10^{-14} cm² s⁻¹, respectively. A high diffusion coefficient for NC-SPE/Anti-LPS was due to higher transfer of electrons after antibody immobilization, which confirms that the antibody was successfully immobilized on NC-SPE.

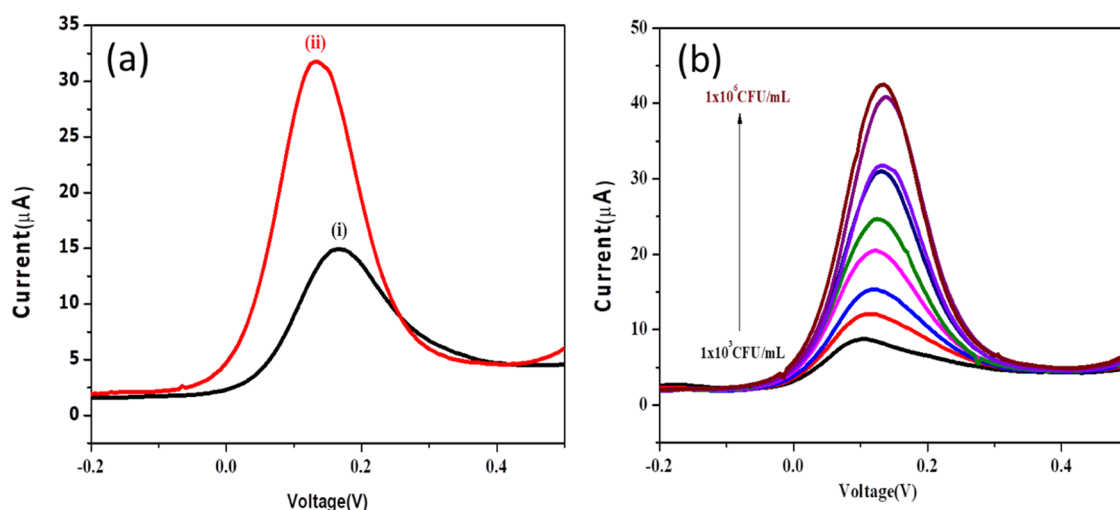


Figure 5. (a) DPV response of (i) NC-SPE and (ii) NC-SPE/Anti-LPS and (b) DPV curve for various concentrations of *E. coli*.

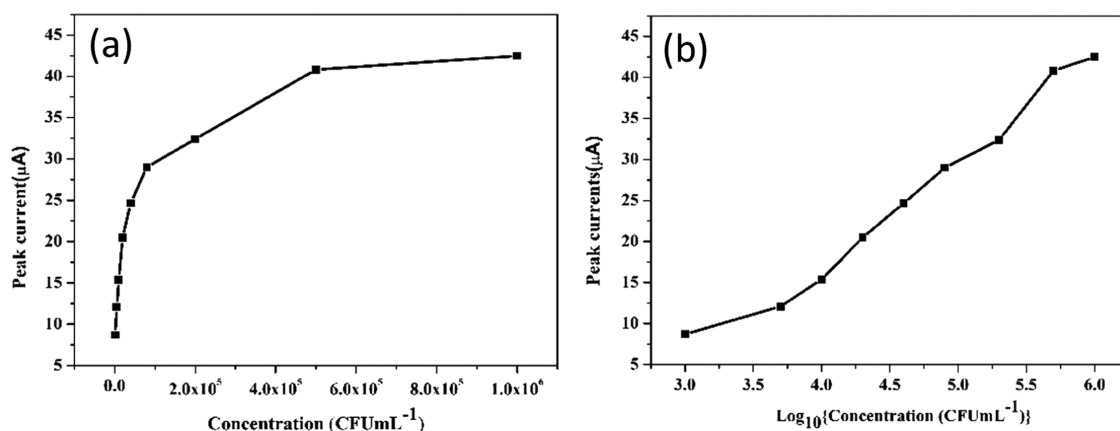


Figure 6. (a) Calibration plot of peak current versus *E. coli* concentration in CFU mL⁻¹ for NC-SPE/Anti-LPS and (b) calibration plots for peak current versus *E. coli* concentration in log₁₀ CFU mL⁻¹.

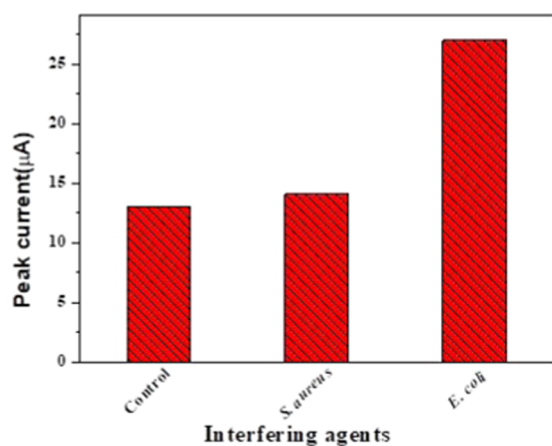


Figure 7. Selectivity bar graph of *E. coli* (Gram-negative) and *S. aureus* (Gram-positive).

The surface concentration of ionic species for NC-SPE and NC-SPE/Anti-LPS was obtained from the Brown–Anson model³⁴

$$\Upsilon^* = \frac{4RTI_p}{n^2F^2A\nu} \quad (6)$$

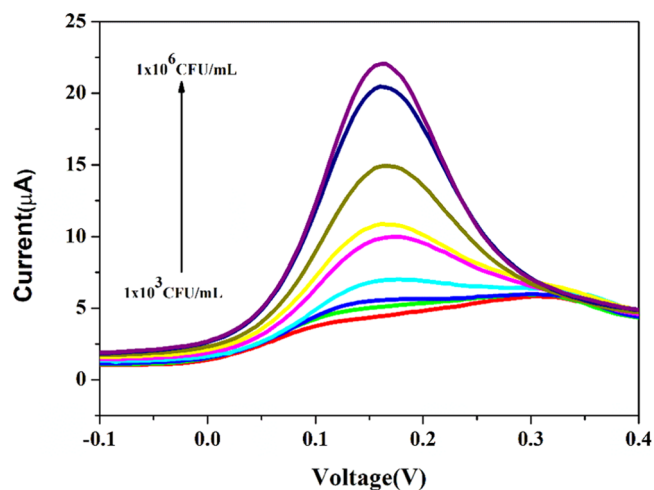


Figure 8. DPV response of milk samples spiked with *E. coli* having different concentrations.

Υ^* is the surface concentration of ionic species, R is the universal gas constant ($8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is room temperature (25°C), I_p is the maximum peak current in ampere, F is the Faraday constant (96485 C mol^{-1}), A is the area of the electrode (0.12 cm^2), n is the number of electrons

Table 1. Binding Affinities between LPS and the Antibody with a Nanoparticle Complex

target receptor	binding affinity (kcal mol ⁻¹)	distance from rmsd lb	distance from rmsd ub
LPS (polysaccharide) _NP	-3.9	0.78	1.24
(1CFV)_NP	-1.9	0.0	0.0

(1), and ν is the scan rate (V s⁻¹). The surface concentrations of ionic species for NC-SPE and NC-SPE/Anti-LPS were found to be 1.3×10^{-8} and 3.3×10^{-8} mol cm⁻², respectively. The higher surface concentration of NC-SPE/Anti-LPS indicates more binding sites available for antigen/*E. coli* binding.

Similarly, differential pulse voltammetry studies of (i) NC-SPE and (ii) NC-SPE/Anti-LPS were carried out to understand the electrochemical process further. During DPV measurements, the change in current was plotted as a function of potential. It was evidenced from Figure 5a that current increases (31.7 μ A) significantly for the NC-SPE/Anti-LPS immunoelectrode, which confirms the binding of the anti-LPS antibody in the electrode. After immobilization of anti-LPS, a slight left shift in peak potential was observed, indicating facile electron transfer at the surface of the electrode. The peak current increases with the increasing concentration of *E. coli* (in CFU mL⁻¹), which indicates that the surface of the electrode is covered with antigen–antibody complexes (Figure 5b).

A calibration curve shows a sharp increase in peak current, which was due to the interaction of *E. coli* with more available binding sites on the anti-LPS antibody on NC-SPE/Anti-LPS (Figure 6a). A log₁₀ (*E. coli* concentration) versus peak current (Figure 6b) was plotted to find the linear relationship. The calibration curve became almost linear in the concentration range of 1×10^3 to 8×10^4 CFU mL⁻¹. The sensitivity of the electrode was estimated from the slope of the calibration curve (Figure 6b). The sensitivity was found to be 11.04 μ A CFU⁻¹ mL⁻¹. The LOD was found to be 2 CFU mL⁻¹ with a 1×10^3 to 8×10^4 CFU mL⁻¹ linear range of detection. ZnO–CuO NC is reported for the first time for fabricating an immunosensor for *E. coli* based on electrochemical detection. The infectious dose for *E. coli* as low as 10 bacterial cells was reported.^{35,36} This was probably due to the large surface area

Table 2. Interactions Formed between the Antibody and NC Complex

receptor–ligand complex	types of interactions formed			
	hydrogen bonds		metal ion interactions	
	residue	amino acid	residue	amino acid
antibody–ZnO–CuO	50L 111H	LYS GLN	48L	SER

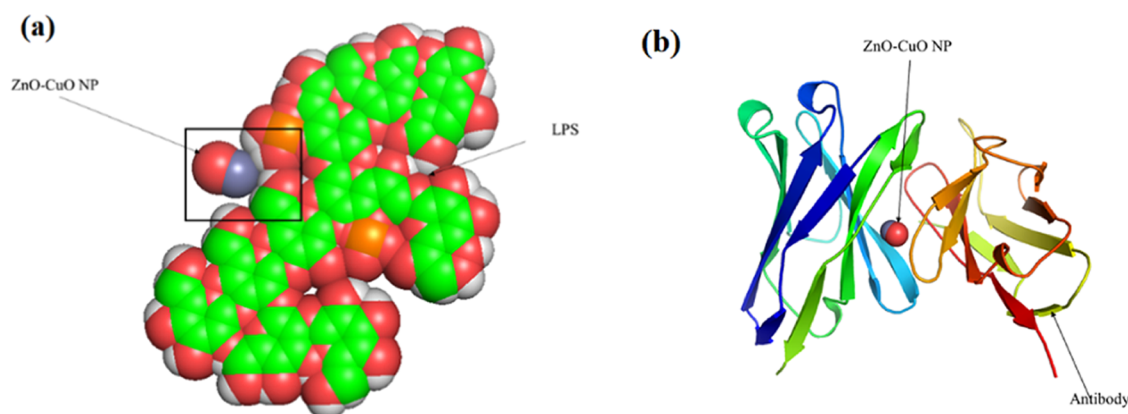
of ZnO–CuO NC, which enhances the generation of reactive oxygen species and thus enhances the antibacterial effect.²³

3.5. Specificity Studies. Cross-reactivity for antibodies is an important concern for immunosensors. To assess the selectivity, interference studies were conducted with *E. coli* and *S. aureus* at a concentration of around 1×10^6 CFU mL⁻¹ cells along with the control. The DPV curve was measured for all of the three samples. The results for an interfering agent were found close to those for the control, indicating the selectivity of the immunoelectrode for *E. coli*. It is also clear in Figure 7 that the maximum rise in the peak current was obtained for *E. coli*.

3.6. Testing the Immunosensor with Real Samples. *E. coli* is often food-borne and can also be found in milk samples. To test the sensitivity of the fabricated immunosensor, various concentrations of *E. coli* were spiked in a milk sample to simulate the real-life milk sample. The DPV response obtained for milk samples spiked with *E. coli* shows a similar trend as in PBS, that is, the *E. coli* concentration is directly proportional to the peak current (Figure 8). This indicates the possible use of the immunosensor in the detection of *E. coli* in milk samples.

3.7. Molecular Docking Studies. Our results showcase that the antibody (PDB ID: 1CFV) has a greater and better affinity for the nanocomposite ZnO–CuO when compared to LPS–ZnO–CuO NP. Table 1 summarizes the docking scores, binding affinity (kcal mol⁻¹), and RMSD for both the complexes. Figure 9 showcases the docked complex. We observed a rigid surface binding of the nanoparticle composite to our target receptor LPS. This clearly proves why the binding affinity is low and the root-mean-square deviation (RMSD) scores are also not optimum.

3.7.1. Atomic Charge Computation. The electronegativity equalization method (EEM) is a fast approach for charge calculations. We observed that LPS with NP had a maximum charge of 62.02745 and a minimum charge of -62.02745, while the antibody against the LPS of *E. coli* (PDB ID: 1CFV)

**Figure 9.** Docked nanoparticle composite (ZnO–CuO) with *E. coli* strain O157: (a) LPS and (b) antibody (PDB ID: 1CFV).

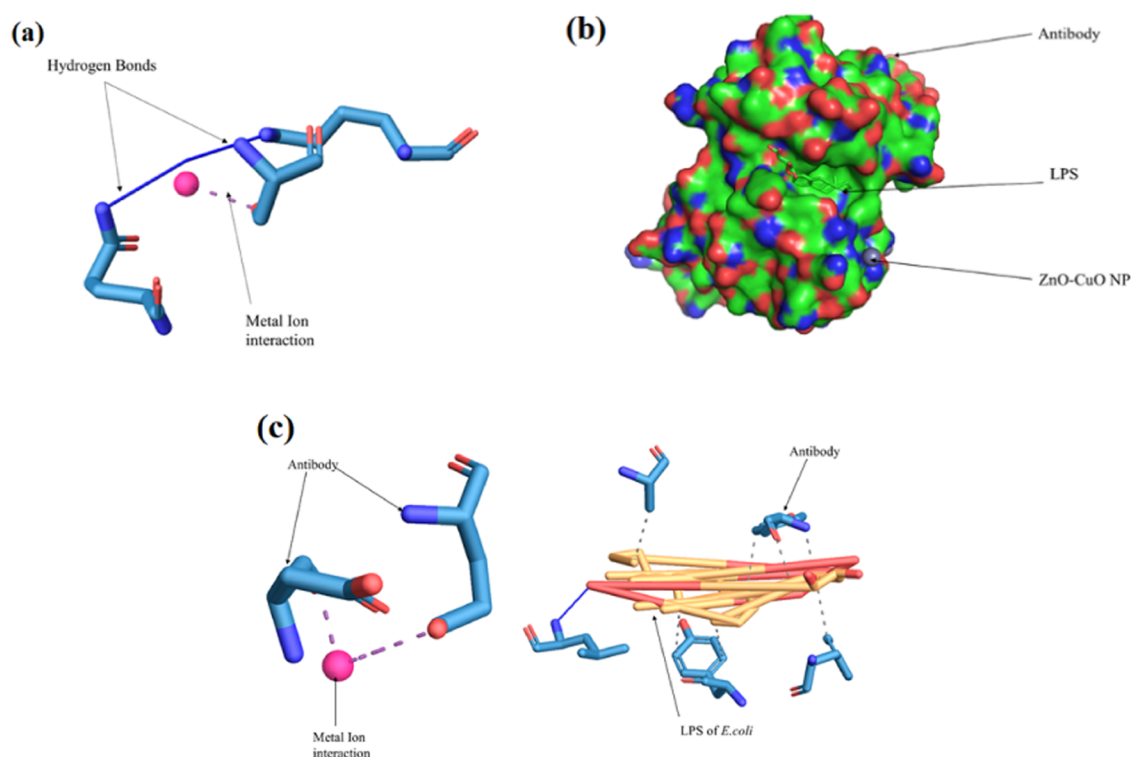


Figure 10. (a) Antibody–NC interactions. (a) Blue lines indicate two hydrogen bonds, while the pink lines indicate the formation of a metal ion interaction, (b) sandwiched antibody–NP–LPS superimposed structure and (c) interactions formed separately between the antibody and NP and the antibody and LPS of *E. coli*.

Table 3. Interactions Formed for the Sandwiched Structure: Antibody–NP–LPS of *E. coli*

receptor–ligand complex	types of interactions formed			
	hydrogen bonds		metal ion interactions	
	residue	amino acid	residue	amino acid
antibody–ZnO–CuO			98L	HIS
antibody–LPS of <i>E. coli</i>	hydrogen bonds		hydrophobic interactions	
	101L	LEU	33H	TYR
			33H	TYR
			33H	TYR
			50H	ALA
			101H	TYR
			104H	TYR
			104H	TYR

with NP had a minimum charge of -1.94071 and a maximum charge of $+1.94071$.

3.7.2. Interaction Analysis. The antibody–NP complex was selected for an interaction analysis using PLIP. It is evident that the complex has formed two hydrogen bonds with residues lysine (50L) and glutamine (111H) and a strong side-chain metal ion interaction with serine (48L). Table 2 shows the number of interactions formed.

Figure 10a depicts how the antibody and NC are interacting with each other. The superimposition of the trio, i.e., the antibody (PDB ID: 1CFV), the nanocomposite (ZnO–CuO), and the LPS of *E. coli*, was further subjected to an interaction analysis. Two different interaction scenarios of the antibody (PDB ID: 1CFV) were observed, one was with ZnO–CuO and the other with the LPS. Figure 10b displays the superimposed structure of the antibody–NC–LPS, while

Figure 10c displays the types of interactions formed separately between the antibody and NC and the antibody and the LPS. Only a single metal ion interaction was formed with the histamine residue (98L) of the NP with the antibody (PDB ID: 1CFV), while only a single hydrogen bond was formed with the leucine residue (101L), with seven hydrophobic interactions of the lipopolysaccharide of the *E. coli* showing strong affiliations (Table 3).

The above interaction analysis indicates that the antibody (PDB ID: 1CFV) strongly binds with the LPS of *E. coli* when compared to the antibody with ZnO–CuO NP. The greater number of hydrophobic interactions is important for protein folding, which in turn is essential for keeping a protein complex stable and biologically active as it reduces surface tension in the protein, further reducing formation of interactions with water molecules present in the environment. Antibody–LPS interactions are stronger when immobilized with a nanoparticle composite (ZnO–CuO). This can also be correlated to electrochemical characterization results, where a higher peak current was observed after antibody immobilization.

4. CONCLUSIONS

We have reported a quick and efficient co-precipitation method for the synthesis of ZnO–CuO NC. The NC was successfully characterized using various techniques such as XRD, FESEM, FTIR spectroscopy, and Raman spectroscopy. The NC was coated on gold electrodes through the screen-printing technique, and then the anti-LPS antibody for *E. coli* was immobilized on the NC surface through physisorption. This platform was used to carry out the electrochemical characterization using CV and DPV. The electrochemical analysis showed the oxidation and reduction properties of ZnO–CuO NC, and the scan rate study confirmed that the

process was diffusion controlled. The response studies were carried out using DPV in the concentration range of 1×10^3 to 1×10^6 CFU mL⁻¹ with linearity up to 8×10^4 CFU mL⁻¹. The high sensitivity and low LOD were obtained for the fabricated sensor. Therefore, it provides one of the best platforms for the electrochemical detection of *E. coli* with low detection limits and high sensitivity. The immunosensor showed selectivity toward *E. coli* and was also able to detect *E. coli* in real milk samples. Computational results revealed that there is a strong correlation between molecular docking studies and electrochemical characterization. The interaction analysis results revealed that antibody–LPS interactions are stronger when immobilized with a nanoparticle composite (ZnO–CuO).

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Notes

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