Electrochemical Immunosensor for Detection of H. pylori Secretory Protein VacA on g-C$_3$N$_4$/ZnO Nanocomposite-Modified Au Electrode

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1. INTRODUCTION

Globally, 50% of the population suffer due to Gram-negative Helicobacter pylori (H. pylori), bacteria that reside in the inner lining of gastric cells. It is spiral-shaped and require very less amount of oxygen for its survival in gastric cells. H. pylori bacteria secrete several virulence factors in host epithelial cells, which in due course of time pave the way to develop gastric cancer. Flagellin, adhesins, and urease primarily support the establishment of bacteria in the host cellular environment, whereas CagA (cytotoxin-associated gene A), VacA (vacuolating cytotoxin A), flagellin, arginase, and superoxide dismutase protect the bacteria from the host immune system(s) and thus facilitate bacterial persistence in host gastric cells.\(^2\) Duodenal ulcer promoting gene A and outer inflammatory protein A along with CagA and VacA are related to the induction of several gastric diseases like peptic ulcers, gastritis, and so forth.\(^7\) However mainly CagA, VacA, and BabA (blood antigen binding protein A) together play an important role in gastric cancer development.\(^5\) Among all these virulence factors, VacA is a major H. pylori secreted multifunctional toxin that assists bacterial colonization in gastric epithelial cells of the host.\(^10\) VacA toxin is required for the survival of bacteria inside the cellular environment of the host by affecting the integrity of gastric epithelial cell(s) through modulation of mucosal ions as well as nutrients flow toward the stomach lumen.\(^11,12\) VacA toxin is the most versatile virulence factor possessing distinct functionalities and diverse receptor(s) present on different cell types that promote bacterial survival and proliferation inside the host’s cellular environment.\(^13\) Hence, VacA was selected as a protein biomarker to detect H. pylori in blood serum. The detection of disease(s)-associated biomarkers is more imperative and has significance in the clinical aspects for early diagnosis of disease(s) such as cancer.\(^14\) It is reported that gastric cancer-related deaths increased extensively over the decades, and H. pylori bacteria are responsible for approximately 70% of gastric cancer(s) cases all over the world.\(^15\) Thus early-stage diagnosis and prognosis of gastric cancer are essential for the prevention of cancer-related deaths.

Although there are several conventional methods present that includes invasive as well as noninvasive detection of H. pylori in gastric cells, they have some drawbacks such as more time consumption, high cost, and short shelf life.\(^19-22\) The emergence of the implementation of biosensors as an analytical tool in the clinical detection of disease(s) interestingly plays a significant role, especially in the biomarker-based diagnosis of cancer.\(^23\) Biosensors emerge as an analytical technique that exhibits high sensitivity and lower cost. Among different types of biosensors, the immunochemical reaction-based electrochemical biosensor is considered as the most sensitive one due to its...
simple preparation with a small sample volume, fast driven analysis, miniaturized disposable devices with precise experimental measurement, and automation.\textsuperscript{24–26} Electrochemical immunosensors are based on antibody-based affinity assays where an analyte (antigen/hapten) is detected by its binding with a region of an antibody. This binding event is responded by an electrochemical transducer which converts the electric response into amplified output signals. The electrochemical reaction in a biosensing surface typically generates a measurable signal in the form of current, conductivity, or potential. Electrochemical-based biosensors can be exploited to achieve a very low limit of detection (LOD) in immunoassays. An ideal electrochemical immunosensor should enable high sample loading and long-term durability/reproducibility without affecting biological activity.\textsuperscript{27,28} Recently, immunosensors gained much attention for clinical diagnosis as they can detect specific interaction between the target antigen and antibodies that form a stable immunocomplex. The antigen–antibody interaction generates measurable electrical signals in response to biomolecule concentration changes. One component, that is, antibody, is covalently attached to the sensing platform coated with nanomaterials and other interactants, that is, analytes or antigens, and are passed over the sensing surface in solution. Electrochemical immunosensors were recently developed to detect SARS-CoV-2 virus which is responsible for the current pandemic. This point-of-care device was based on the multichannel electrochemical immunosassay.\textsuperscript{29,30} Electrochemical immunosensors were also reported for the detection of \textit{Mycobacterium tuberculosis} secreted protein CFP10-ESAT6 that is responsible for tuberculosis,\textsuperscript{31} prostate cancer-specific biomarkers,\textsuperscript{32,33} and other infectious diseases.\textsuperscript{34}

In the fabrication of immunosensors, the most crucial step is the immobilization of biomolecules such as antibodies. Thus, nanomaterials are preferred for the immobilization of biomolecules as a substantial matrix. Biosensors in combination with nanomaterials provide tunable surface modification ability due to the high surface-to-volume ratio to empower the loading capacity of the sensing platform. Nanomaterials exhibit several properties like electrical, thermal, optical, and catalytic with strong mechanical strength. These properties offer promising opportunities for the development of a biosensor to detect infectious disease biomarkers. Due to the nanomaterial’s large surface area, the possibility of immobilization of bioreceptors in enhanced quantity and low volume is increased several times. Various such types of nanomaterials have been used for this purpose, for example, gold, graphene, nanotubes, nanodiamonds, quantum dots, and some polymeric nanoparticles.\textsuperscript{34,35}

In this study, a nanocomposite comprised of graphitic carbon nitride (also called GCN or g-C\textsubscript{3}N\textsubscript{4}) nanosheets decorated with zinc oxide (ZnO) nanorods has been synthesized by using the thermal condensation method. The g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite evidently showed increased surface area, more active sites for trapping molecules, better light absorbing capacity, and reduced band gap.\textsuperscript{36,37} The coupling or doping with metal-oxide nanomaterials can improve the electric conductivity of g-C\textsubscript{3}N\textsubscript{4}. Furthermore, the nanostructured ZnO is widely used due to its superior conductivity. It is also known for its high chemical and photo stabilities as well as its high electrochemical coupling coefficient. ZnO is an inorganic electrochemically active transition metal oxide that efficiently transports electrons. The most suitable morphologies of ZnO are nanorods, nanobelts, and nanowires due to their higher surface-to-volume ratios and excellent transfer of electrons to the length direction.\textsuperscript{38} However, some intrinsic parameters of ZnO restrict its wide-scale applications. To remove the restriction, a carbon-based nanomaterial, especially g-C\textsubscript{3}N\textsubscript{4}, is used to achieve better performance in sensing applications. ZnO and g-C\textsubscript{3}N\textsubscript{4} show pronounced electrical and catalytic properties due to the remarkable synergy between them. The well-known conjugated p-structure of g-C\textsubscript{3}N\textsubscript{4} and the combination of ZnO heterostructure may enhance the electron-transfer process, and thus this nanocomposite can be employed as an ideal candidate for electrochemical sensing applications.\textsuperscript{39} So in this study, the electrical conductivity of the synthesized nanocomposite of g-C\textsubscript{3}N\textsubscript{4}/ZnO for detection of \textit{H. pylori}-specific toxin is explored. In addition, g-C\textsubscript{3}N\textsubscript{4}/ZnO is easily available, cost-effective, and nontoxic and hence can be used effectively in electrochemical sensors.\textsuperscript{40} This study reports g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite that has been utilized as the matrix for fabrication of a label-free and facile immunosensor to detect VacA toxin of \textit{H. pylori}. Furthermore, the gold electrode or Au-ET was modified with the nanocomposite to provide enhanced surface properties to the sensing platforms such as a large surface-to-volume ratio, stability, biocompatibility, and dispersibility. The nanocomposite-coated Au-ET served as an improved biocompatible platform for the immobilization of VacA antibodies (VacA Abs). Electrochemical studies performed in the current work involve cyclic voltammetry (CV), differential pulse voltammetry (DPV), and impedance techniques. Moreover, the proposed g-C\textsubscript{3}N\textsubscript{4}/ZnO-coated electrochemical immunosensor delivers good stability, improved sensitivity, and, more importantly, a low LOD for \textit{H. pylori} detection.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. Melamine (C\textsubscript{3}H\textsubscript{6}N\textsubscript{3}) and zinc acetate dihydrate [Zn(CH\textsubscript{3}COO)\textsubscript{2}·2H\textsubscript{2}O] were procured from LOBA CHEMIE Pvt. Ltd, Mumbai, India and Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India. VacA Abs and antigens were obtained from the Max von Pettenkofer Institute, Munich, Germany. The 23-carat gold wire which was 0.7 mm thick and of 0.3 cm geometric area was purchased from NOIDA, India. Potassium ferricyanide [K\textsubscript{3}Fe(CN)\textsubscript{6}] and potassium ferrocyanide [K\textsubscript{4}Fe(CN)\textsubscript{6}] were procured from Fisher Scientific, and (3-dimethylaminopropyl)-3-ethylcarbodiimide(EDC)-N-hydroxysuccinimide ( NHS) was purchased from Sisco Research Laboratory.

2.2. Instruments. All electrochemical experiments were performed by using a Biologics SP-150 potentiostat, which is a three-electrode system consisting of auxiliary, counter, and working electrodes. In this system, Ag/AgCl is used as an auxiliary, platinum as a counter, and gold wire as a working electrode. The synthesized nanocomposite was characterized by FESEM to evaluate the surface morphology using a FEI Quanta 3D FEG microscope. The crystallinity was analyzed with X-ray diffraction (XRD) using a Rigaku Smart Lab (2θ range: 5 to 40°) diffractometer. FTIR spectra were recorded from a Bruker Tensor 37 instrument in the range of 500–4000 cm\textsuperscript{-1}. Double-distilled water was used throughout the experiment.

2.3. g-C\textsubscript{3}N\textsubscript{4} Synthesis. g-C\textsubscript{3}N\textsubscript{4} was obtained through the thermal condensation method. Initially, fine melamine powder (5 g) was taken into a ceramic crucible and kept at a temperature of 550 °C with a heating rate of 5 °C/min in a 32293 https://doi.org/10.1021/acsomega.2b03627 ACS Omega 2022, 7, 32292–32301
muffle furnace. Finally, the yellow bulk g-C$_3$N$_4$ was obtained after cooling the furnace at room temperature.

2.4. Synthesis of g-C$_3$N$_4$/ZnO Nanocomposite. Figure S1 shows the synthesis of g-C$_3$N$_4$/ZnO nanocomposite. Zinc acetate dihydrate and g-C$_3$N$_4$ powder in the ratio of 1:1 were finely ground using a mortar and pestle for 45 min. After that, the fine mixture was moved into a ceramic crucible to be kept in the muffle furnace for 4 h at 275 °C with a 4 °C/minute heating rate. The synthesized g-C$_3$N$_4$/ZnO nanocomposite was further ground into a fine powder. In the present study, the g-C$_3$N$_4$/ZnO nanocomposite was prepared in the ratio of 1:1. It has been seen that g-C$_3$N$_4$ consists of remarkable superiority over graphene due to its more active sites. However, g-C$_3$N$_4$ possesses poor electrical conductivity which restricts the analytical performance of the material. Therefore, the ZnO loading was necessary to enhance the conductivity of the nanocomposite, and with the increase in ZnO proportion in the g-C$_3$N$_4$/ZnO nanocomposite, the conductivity of the nanocomposite will be increased which will have a huge impact on the analytical performance.

2.5. Electrochemical Deposition of g-C$_3$N$_4$/ZnO Nanocomposite on the Au-ET Surface. Before starting the electrochemical experiments, the Au-ET is primarily activated for 10 min in freshly prepared piranha solution, that is, 98% H$_2$SO$_4$ and 30% H$_2$O$_2$, in a 3/1 v/v ratio. Piranha solution, a strong oxidizing agent, is able to remove impurities such as organic residues. After that, the Au-ET is washed again with ultrapure water and dried in air. Then, the activated Au-ET is submerged in a solution of nanocomposite for electrode-deposition through the CV technique at 50 mV/s scan rate, 10 cycles, and -0.6 to 1.4 V potential range. The modified electrode is then washed with distilled water.

2.6. Immobilization of VacA Antibodies Over g-C$_3$N$_4$/ZnO@Au-ET. Subsequently, EDC-NHS chemistry was used for covalent attachment of VacA Abs to the g-C$_3$N$_4$/ZnO nanocomposite. EDC-NHS activates the carboxylic group at the Au-ET surface. The g-C$_3$N$_4$/ZnO/Au-ET was thoroughly rinsed with ultrapure water. Afterward, the electrode was immersed in 100 mM MES buffer [2-(N-morpholino)-ethanesulfonic acid] of pH 5 that contains 1:1 ratio of 2 mM EDC and 5 mM NHS for 1.30 h at room temperature. After rinsing, the Au-ET surface was incubated with 1:100 dilution of VacA Abs for 12 h at 4 °C. After 12 h, the electrode was immersed in 0.1 M PBS buffer of pH 7.5 to remove unbound antibodies. The fabricated immunosensor was processed further for blockage of nonspecific binding sites with the help of PBS and BSA (1%) solution for 1 h.
Ultimately, the immunosensor was further washed, dried, and kept at 4 °C for further electrochemical characterization.\textsuperscript{43,44}

2.7. Electrochemical Measurement. The three-electrode system potentiotstat was employed for electrochemical measurements through CV, electrochemical impedance spectroscopy (EIS), as well as DPV. The 0.1 mM K\textsubscript{3}[Fe(CN)\textsubscript{6}] and K\textsubscript{4}[Fe(CN)\textsubscript{6}] containing electrolyte was used for all electrochemical measurements. EIS was performed within the 10\textsuperscript{5} to 10\textsuperscript{−1} Hz frequency range. For impedance spectra, the Nyquist plot was studied in which the curve consists of a semicircle and a straight line. The X-axis represents the real part, whereas the Y-axis represents the imaginary part in the Nyquist plot. The potential ranging from −2 to 2 V was taken for the measurement of DPV.\textsuperscript{45}

A schematic representation is shown in Scheme 1 for the step-wise processes involved in the fabrication of the VacA Abs/g-C\textsubscript{3}N\textsubscript{4}/ZnO/Au-ET immunosensor.

2.8. Real Sample Detection. In this study, to observe the clinical parameters and the validation of the fabricated biosensor, we acquired 20 human serum samples of anonymous donors from Bio Diagnostics Lab Rohini, New Delhi, India. Afterward, the fabricated immunosensor was also tested with serum samples spiked with the VacA antigen.

3. RESULTS AND DISCUSSION

3.1. Morphological and Crystallographic Characterization. 3.1.1. FESEM and EDAX. Figure 1A,B shows the FESEM image of g-C\textsubscript{3}N\textsubscript{4} and g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite, respectively. Bulk g-C\textsubscript{3}N\textsubscript{4} as shown in Figure 1A consists of layered structures which are agglomerated to each other, while the g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite shows the ZnO nanorods onto the surface of bulk g-C\textsubscript{3}N\textsubscript{4} in Figure 1B. It shows that bulk g-C\textsubscript{3}N\textsubscript{4} is densely packed with ZnO nanorods. During calcination, the formation of ZnO nanorods with different lengths and widths along with nearly regular and elongated hexagonal structures were thoroughly occupied onto the surface of layered g-C\textsubscript{3}N\textsubscript{4}.\textsuperscript{46}

Furthermore, the EDAX mapping shows the composition as well as the distribution of the atoms present in the g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite in Figure 2. The addition of ZnO in the g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite was confirmed from the EDAX plot. The inset in the EDAX shows the atomic % and weight % of individual atoms in the g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite which gives strong evidence for the presence of major atoms such as C, N, O, and Zn. The atomic % of Cu present in g-C\textsubscript{3}N\textsubscript{4}/ZnO was due to the Cu grids onto which the sample was prepared.

3.1.2. XRD. The XRD patterns confirmed the phase and composition of the g-C\textsubscript{3}N\textsubscript{4}/ZnO nanocomposite. Figure 3 shows the XRD peaks of g-C\textsubscript{3}N\textsubscript{4}/ZnO observed at 2θ values of 12.3, 27.5, 31.75, 34.42, 35.95, 47.42, 56.54, 62.81, 66.86, 67.81, and 69.06° corresponding to (100), (002), (100), (002), (101), (102), (110), (110), (112), and (201), confirming the crystalline structure of g-C\textsubscript{3}N\textsubscript{4}/ZnO that well-matched and identified with heptazine units (JCPDS no. 87−1526), whereas wurtzite hexagonal structures (JCPDS no 36−1451) confirms the crystalline nature of prepared nanocomposite.\textsuperscript{36,46}

3.1.3. FTIR. Figure 4 shows the typical FTIR spectrum. The sharp FTIR peaks appearing at 557 and 814 cm\textsuperscript{−1} correspond to the Zn–O stretching bond and triazine units. The FTIR adsorption bands in the range from 1200−2000 cm\textsuperscript{−1} indicate the CN heterocyclic ring’s associated stretching modes. The sharp adsorption peaks seen at 2341 and 3741 cm\textsuperscript{−1} appear due to physically adsorbed CO\textsubscript{2} and H\textsubscript{2}O from the atmosphere. Furthermore, the NH group’s associated stretch-
ing modes can be observed at absorption band ranging from 2900 to 3400 cm\(^{-1}\).

### 3.2. Electrochemical Characterization of Fabricated Immunosensing Platform.

Figure S2 demonstrates the electrodeposition of the nanocomposite over Au-ET at 50 mV/s scan rate by adjustment of potential range from −0.6 to 1.4 V. Interfacial concentration of ions has been adjusted in the electrochemical deposition of the nanocomposite by reduction of precursor ions present in the electrolyte solution. As characterized by the SEM image, the ZnO nanorods thoroughly occupied the g-C\(_3\)N\(_4\) surface; therefore, it could be predicted that ZnO nanorods exhibit a strong influence on conductivity. CV curve depicts the characteristics of current features for electrodeposition of the nanocomposite at −0.2 and 0.45 V. The peak at −0.2 V indicates the adsorption of reducing protons.

Interface properties of the surface-modified Au-ET were detected to achieve oxidation and reduction peaks through the CV technique. Figure 5A shows the CV curves of Au-ET (bare), g-C\(_3\)N\(_4\)/ZnO/Au-T, and VacA Abs@g-C\(_3\)N\(_4\)/ZnO/Au-ET at 50 mV/s scan rate with a potential range from −0.6 to 0.6 V. In the first step, current response was taken for the bare electrode which showed slightly compressed redox peaks. The value of the oxidation peak current was gradually increased after deposition of the nanocomposite on the bare electrode which demonstrated the good electrical conductive nature of the nanomaterials. The CV curves expose the electron transfer rate and redox peak current of the g-C\(_3\)N\(_4\)/ZnO nanocomposite which are significantly higher as compared to the bare electrode. Once VacA Abs were immobilized on the nanocomposite-coated electrode interface, the current response gets down. This behavior of the electrode showed the fact that VacA Abs block the electron transfer by the formation of a layer that interrupts redox reactions and shows the nonconductive characteristic of the VacA protein.

The EIS technique is also important to determine the resistance on the electrode material and also between the electrode and the electrolyte.\(^{49}\) The \(R_{ct}\) value (charge-transfer resistance) of the nanocomposite-decorated Au-ET interface was monitored. In Figure 5B, impedance curves are reported for bare Au-ET, g-C\(_3\)N\(_4\)/ZnO/Au-ET, and VacA Abs@g-C\(_3\)N\(_4\)/ZnO/Au-ET in 0.1 M potassium ferricyanide and potassium ferrocyanide electrolyte solution. The semicircular graph was observed which is attributed to the restricted electron transfer at the high-frequency region and diameter that is \(R_{ct}\) value equivalent. The graph indicates bare Au-ET owing to higher electric conductivity of g-C\(_3\)N\(_4\)/ZnO. However, the \(R_{ct}\) values are increased significantly after deposition of antibodies on g-C\(_3\)N\(_4\)/ZnO/Au-ET which reflects their effective immobilization. The deposited nanocomposite had higher conductivity and showed a lower resistance due to surface variation caused by resistance in transmitting charges. The immobilization of VacA Abs on g-C\(_3\)N\(_4\)/ZnO/Au-ET further blocked the electron transfer and resulted in higher resistance. Furthermore, the VacA-Abs-modified electrode encourages electron obstruct and mass transfer. It also increases the resistance by insulating the conductive surface of the electrode via inhibition of oxidation and reduction reactions. This result further confirms the successful and effective construction of the immunosensor.

### 3.3. Analytical Performance of Immunosensor.

DPV was utilized for the analysis of VacA antigen concentrations ranging from 0.1 to 12.8 ng mL\(^{-1}\) on the fabricated immunosensor. Figure 6A exhibits the catalytic reduction peak current. The study was carried out in the potassium

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**Figure 4.** FTIR plot of g-C\(_3\)N\(_4\)/ZnO nanocomposite.

**Figure 5.** (A) CV curves and (B) Nyquist Plot in 0.1 mM \([\text{Fe(CN)}_6]^{3-/4-}\) electrolyte at 50 mV s\(^{-1}\) of bare Au-ET, g-C\(_3\)N\(_4\)/ZnO/Au-T, and VacA Abs@g-C\(_3\)N\(_4\)/ZnO/Au-ET.
ferrocyanide/ferricyanide electrolyte and showed that current linearly decreased with an increase in the antigen concentration, that is, the highest current was obtained at 0.1 ng mL$^{-1}$ and the lowest at 12.8 ng mL$^{-1}$ concentration. The interaction of the antigen with immobilized antibodies on a modified electrode surface formed the antigen–antibody complex which hinders the inert kinetics to transmit electrons in the electrolyte mediator at the optimum concentration. These results depict that the limit of detection is 0.1 ng mL$^{-1}$.

Figure 6B exhibited a standard plot based on the result obtained in the DPV study of the fabricated immunosensor. A linear range in the calibration plot is shown among different current ranges and various VacA Abs concentrations between 0.1 to 12.8 ng mL$^{-1}$. The sensitivity of the developed immunosensing electrode has been calculated as 0.3 $\mu$A ng mL$^{-1}$. Linear correlation of current and concentration is expressed through an equation as $y = -8.0953x + 163.55; R^2 = 0.9329$.

3.4. Biosensing Response of Immunosensor at Different Scan Rates. The CV plots of Figure 7A show the study of the biosensing response of modified electrodes observed for various scan rates ranging from 10 to 100 mV/s with the applied potential of −0.4 to 0.4 V. The scan rate graph shows a diffusion reaction of electron transfer based on a quasi-reversible process. Symmetry can be seen in oxidation and reduction peaks at a similar potential. With the increase in the range of scan rate, an enhancement of peak has been observed. Similarly, Figure 7B shows the calibration graph between the scan rate’s square root and the redox peak current. It can be observed through the calibration curve that the scan rate’s square root is directly proportional to the redox current. The calibration plot between the square root of the scan rate and current is as follows

$$y = 0.0083x + 0.6858; R^2 = 0.8974 \text{ (Ipa)}$$

$$y = -0.0089x - 0.8659; R^2 = 0.9232 \text{ (Ipc)}$$

3.5. pH Effect on Modified Electrode. In Figure 8, the electrochemical behavior of the fabricated electrode at a distinct pH range, that is, 5.5 to 9, can be observed. For pH optimization, an experiment was carried out in electrolytes with different pH solutions. The current was observed to increase from the increase in pH from 5.5 to 7 pH and then decrease subsequently from 7 to 9 pH. This shows that the electrode functions best at neutral pH, that is, at 7, due to loss
in protein activity at extremely acidic and basic surrounding conditions. So, pH 7 is selected for the proper functioning of the immunosensor by maintaining its physiological parameters.

### 3.6. Analysis of Selectivity and Stability

The fabricated electrode was studied in presence of various interfering compounds in their physiological range like 0.05 mM ascorbic acid, 5 mM glucose, and 0.2 mM uric acid, whereas *H. pylori* specific antigens BabA and CagA concentrations were taken as 1 ng mL$^{-1}$. The study was conducted in the presence of 0.1 ng mL$^{-1}$ VacA antigen. Figure 9 shows the selectivity of the fabricated immunosensor in the presence of various types of interferents. The results demonstrate that after adding the interferents in the presence of VacA, less than 15% variation in current was observed. Changes in the DPV currents were altered slightly, but no prominent variations have been seen as compared to VacA only. The developed immunosensor showed high specificity (87.03%) and accuracy as compared to conventional methods (Table 1).$^{31,52}$ Therefore, the study showed the high selectivity of the fabricated immunosensor for VacA detection as the whole assay was performed in the presence of approximately 5 times higher concentration of glucose, ascorbic acid, and uric acid.

The reproducibility of the present immunosensor was also observed with five independent electrodes to evaluate the consistency. The consistency is observed by comparing the coefficients of variation of five equally prepared working electrodes with 0.1 ng mL$^{-1}$ VacA concentration. In Figure 10, the current response varies very slightly between interassay, and thus it shows good repeatability of the presented immunosensor.

### 3.7. Real Sample Analysis

Further, the developed immunosensor was investigated with blood samples to demonstrate the clinical application. The accuracy of the fabricated immunosensor in the presence of various types of interferents. The results demonstrate that after adding the interferents in the presence of VacA, less than 15% variation in current was observed. Changes in the DPV currents were altered slightly, but no prominent variations have been seen as compared to VacA only. The developed immunosensor showed high specificity (87.03%) and accuracy as compared to conventional methods (Table 1).$^{31,52}$ Therefore, the study showed the high selectivity of the fabricated immunosensor for VacA detection as the whole assay was performed in the presence of approximately 5 times higher concentration of glucose, ascorbic acid, and uric acid.

The reproducibility of the present immunosensor was also observed with five independent electrodes to evaluate the consistency. The consistency is observed by comparing the coefficients of variation of five equally prepared working electrodes with 0.1 ng mL$^{-1}$ VacA concentration. In Figure 10, the current response varies very slightly between interassay, and thus it shows good repeatability of the presented immunosensor.

### Table 1. Comparison of Analytical Parameters of Different Detection Methods with the Presented Immunosensor

| Detection method       | Accuracy | Specificity | Time consumption |
|------------------------|----------|-------------|-----------------|
| histopathology         | 95.3%    | 77.8%       | about 7 days    |
| PCR                    | 94.5%    |             | 24 h            |
| serology               | 86%      | 60%         | more than 3 h   |
| stool antigen test     | 80.2%    | 86.7%       | 1–4 days        |
| rapid urease test      | 73.6%    | 85%         | 40 min          |
| presented immunosensor | 96.2%    | 87.03%      | 10–15 min       |

The fabricated immunosensor was stored at 4 °C to investigate its stability. The stability was checked after every 3 days to demonstrate the peak current. The current was reduced by only 6% after 15 days of storage which illustrates the long-term durability of the immunosensor. Additionally, in Table 2, the developed immunosensor has been compared with previously reported biosensors which also proved that the developed immunosensor has high sensitivity with a low LOD.

### 3.7. Real Sample Analysis

Further, the developed immunosensor was investigated with blood samples to demonstrate the clinical application. The accuracy of the...
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03627.

Table 2. Comparative Analysis of Previously Reported Biosensors with the Presently Developed Immunosensor [36–41]

| type of biosensors                  | biomarkers used | LOD    | linear range | references |
|-------------------------------------|-----------------|--------|--------------|------------|
| electrochemical DNA biosensor       | DNA             | $8.3 \times 10^{-4}$ M | 8.9–22.2 μM | [53]       |
| electrochemical DNA biosensor       | DNA based       | $0.17 \times 10^{-3}$ M | 0.3–240 nM/L | [54]       |
| microfluidic based immunosensor     | *H. pylori* antigen | 10 ng  | 10–1000 ng   | [55]       |
| FRET-based optical biosensor       | DNA             | $4.5 \times 10^{-5}$ M | 10–200 nM   | [56]       |
| immunosensor                        | BabA antigen    | 0.2 ng mL$^{-1}$ | 0.2–20 ng mL$^{-1}$ | [45]       |
| electrochemical DNA biosensor       | DNA             | 0.15 nM | 0.3 nM–0.24 μM | [57]       |
| electrochemical DNA biosensor       | DNA             | 7.2 nM  | 2.0 to 410 nM | [58]       |
| voltametric immunosensor            | CagA antigen    | 0.2 ng mL$^{-1}$ | 0.2 to 50 ng mL$^{-1}$ | [45]       |
| electrochemical immunosensor        | VacA antigen    | 0.1 ng mL$^{-1}$ | 0.1 to 12.8 ng mL$^{-1}$ | this work  |

Table 3. Recovery of VacA Antigen by Using the Electrochemical Immunosensor

| added concentration (ng mL$^{-1}$) | observed concentration | recovery percentage |
|------------------------------------|------------------------|---------------------|
| 0.1                                | 0.094                  | 96.21               |
| 0.5                                | 0.476                  |                     |
| 1.0                                | 0.968                  |                     |
| 10                                 | 9.885                  |                     |

The observations showed that the immunosensor is very selective for VacA antigen of *H. pylori* and also easy to handle.

4. CONCLUSIONS

In precise, a highly conductive nanocomposite g-C$_3$N$_4$/ZnO was used to construct an electrochemical immunosensor for the detection of a distinct toxin VacA of *H. pylori* bacteria. The thermal decomposition method was exploited to synthesize the g-C$_3$N$_4$/ZnO nanocomposite. The developed immunosensor showed good reproducibility, higher sensitivity (0.3 μA·ng mL$^{-1}$), and favorable stability suggesting that the immunosensor can provide prospective applications in the clinical diagnosis of *H. pylori*. The immunosensor can detect a very low concentration of VacA antigen in a linear range of 0.1 to 12.8 ng mL$^{-1}$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03627.

Schematic representation of the steps involved in the synthesis of the g-C$_3$N$_4$/ZnO nanocomposite and electrodeposition of the g-C$_3$N$_4$/ZnO nanocomposite at 50 mV/s in the potential range of −0.6 to 1.4 V for 10 cycles (PDF)

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Notes

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