Mn_3O_4 tetrahedral with carbonyldiimidazole nanoflower deposition on Laser Scribed Graphene for selective bio-capture.

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Abstract

Dengue fever, a mosquito-borne viral infection, poses a significant global health challenge, particularly in tropical and subtropical regions. The absence of non-effective vaccines and specific treatments underscores the need for advanced diagnostic tools for early detection and management. This study presents a novel biosensor for detecting dengue virus type 4 (DENV-4) by combining carbonyldiimidazole nanoflower (CDI-NF) with Mn_3O_4 on laser-scribed graphene (LSG). Material characterization techniques, including Raman spectroscopy, TEM, XRD, XPS, and FTIR, were employed to confirm the successful integration of Mn_3O_4 and CDI-NF, resulting in a unique 3D flower-like structure. In order to verify the sensing efficiency, a selective DNA sample captured on LSG/Mn_3O_4-CDI-NF was investigated for specifc binding with *Aedes Aegyptii* target DNA through selective hybridisation and mismatch analysis. Electrochemical impedance studies further confirmed sensitive detection of up to 1 fM, where the sensitivity confirmed by large transfer resistance (R_{ct}) before and after hybridization with the a regression coefficient 0.97373. EIS results demonstrated successful surface modifications and the biosensor's specificity in distinguishing between complementary, mismatched, and non-complementary target sequences. The biosensor's ability to differentiate between these sequences highlights its potential for accurate and targeted DENV-4 detection, offering a promising avenue for advancing dengue diagnostics.

Keywords: Electrochemical biosensor; surface functionalization; Laser scribed graphene; Dengue virus

1.0 Introduction

Dengue fever is a mosquito-borne viral infection that poses a significant global health burden. It is transmitted by the bites of infected Aedes mosquitoes, primarily Aedes aegypti and Aedes albopictus. The virus manifests in a spectrum of clinical presentations, ranging from asymptomatic infection to severe dengue, which can lead to life-threatening complications like dengue hemorrhagic fever and dengue shock syndrome. Dengue virus belongs to the Flaviviridae family and comprises four distinct serotypes (DENV-1 to DENV-4). Infection with one serotype provides immunity to that specific serotype but only partial or temporary immunity to others, increasing the risk of severe disease upon subsequent infections [1], [2], [3]. Dengue fever is a significant global health concern, putting a considerable burden on public health systems. It leads to millions of infections and hospitalizations every year. The symptoms of dengue fever are similar to those of the flu and can range from mild to severe. In severe cases, the disease can progress to a life-threatening condition known as dengue hemorrhagic fever (DHF). Dengvaxia is the first approved dengue vaccine, made by Sanofi Pasteur. It uses a weakened yellow fever virus as its base, with parts replaced by pieces of all four dengue virus types. Given in three doses, Dengvaxia seems to work well for people who've had dengue before. However, it can increase the risk of severe dengue in those who never had it before. This might happen because of a process called antibody-dependent enhancement, where existing antibodies can make the infection worse. Because of this safety concern, Dengvaxia's use is limited in some places. Still, it's valuable in areas with lots of dengue, especially where many people have had it before. Scientists are still studying its risks and benefits to find the safest ways to use it [4]. Among the four types of dengue virus, DENV-4 is a particular concern as it poses a significant threat to public health. This type of virus is found in Asia, Africa, and the Americas, adding to the overall burden of dengue fever.

Biosensors, employing techniques such as electrochemical, optical, and piezoelectric methods, have emerged as promising tools for rapid and sensitive detection of dengue viruses and its associated biomarkers. Biocapture devices are gaining popularity and offer rapid diagnosis and timely treatment. Their high accuracy in detecting dengue virus components or antibodies ensures reliable results. The preference for non-invasive or minimally invasive testing methods, combined with their cost-effectiveness and ease of use, makes these devices more accessible in places with limited resources and regions prone to dengue outbreaks. Moreover, these devices play a vital role in disease surveillance and outbreak management, enabling early detection and better response strategies. However, the effectiveness of biosensors for detecting dengue can be improved [5].

The integration of laser-scribed graphene (LSG) into bioelectrodes offers several key advantages for biosensor applications [6]. LSG's exceptional electrical conductivity facilitates rapid electron transfer between the biorecognition element (e.g., enzyme, antibody) and the transducer, enabling fast and sensitive signal detection [7]. Moreover, its high surface area provides ample space for immobilizing a large number of biorecognition elements, enhancing the sensor's sensitivity and detection limit [8]. Additionally, LSG's biocompatibility and chemical stability make it suitable for various biological environments, minimizing interference from background molecules [9]. Furthermore, the laser-scribing process is a simple, cost-effective, and scalable method for fabricating LSG electrodes, making it a promising technology for the development of next-generation, high-performance biosensors.

A possible avenue is the use of manganese (II, III) oxide, commonly known as Mn_3O_4, which has garnered significant attention from researchers due to its wide range of properties and potential applications. By combining Mn_3O_4 with materials such as graphene, nanocomposites with enhanced properties can be created. For instance, when Mn_3O_4 is

incorporated into graphene-based nanocomposites, it has demonstrated improved performance as electrodes in supercapacitors [10]. Mn_3O_4, with its mixed valence states of Mn^{2+} and Mn^{3+}, undergoes reversible redox reactions at the electrode-electrolyte interface during charging and discharging. These reactions involve the transfer of electrons between the Mn ions and the electrolyte, leading to changes in the oxidation states of manganese. This Faradaic process contributes significantly to the overall capacitance of the supercapacitor [11]. As nanotechnology continues to progress, the use of Mn_3O_4 nanoparticles and nanostructures shows great promise in nanotechnology and nanomedicine. Mn_3O_4 can be synthesized in various forms, such as nanorods, nanoparticles [12], and nanocomposites [10]. This versatility enables researchers to customize the material's properties to suit specific applications. Remesh et al. [13], developed a selectively growing nano-octahedral Mn_3O_4 on lignin-derived laser scribed graphene for microsupercapacitor applications. Improved Electrical Conductivity whereas Mn3O4, while possessing good intrinsic conductivity, can be further enhanced by its integration with graphene. Graphene's excellent electrical conductivity provides a conductive network that facilitates electron transport, reducing internal resistance and improving charge transfer kinetics [13]. The resulting composite exhibits high performance, attributed to the synergistic effects between Mn_3O_4 and graphene. Their research opened up new avenues for developing efficient energy storage devices with enhanced capacitance and stability.

Biosensors can significantly benefit from the incorporation of nanomaterials in various dimensions (0D to 3D). Among these, multifunctional hybrid nanostructures have gained significant traction due to their successful application in diverse fields. Hybrid nanomaterials with 3D flower-like morphologies, first reported in 2012 by Zare et al., have emerged as a prominent class of materials. Their hierarchical structure offers several advantages, including a high surface area, excellent catalytic properties, enhanced loading efficiency, and strong adsorption capacity. These factors have fueled the rapid development of these materials. A

wide range of inorganic (e.g., copper, calcium, zinc, iron) and organic components (e.g., enzymes, aptamers, DNA) have been successfully utilized to synthesize these hybrid nanoflowers with diverse morphologies, such as rosette, rhombic, and spherical shapes [14], [15], [16], [17], [18], [19], [20].

A nanoflower refers to a specific type of nanostructure that takes on the shape of a flower at the nanoscale level. It is a three-dimensional arrangement of nanomaterials, characterized by nanoscale petals or branches radiating from a central core, resembling the natural structure of a flower. One such nanoflower is the carbonyldiimidazole-nanoflower (CDI-NF), a bimetallic alloy nanoflower structure known for its outstanding catalytic activity. This material possesses several properties that make it highly promising for various applications. 1,1'-Carbonyldiimidazole (CDI) is an organic compound known for its highly reactive nature as a carboxylating agent. It possesses two acylimidazole leaving groups and hydroxylated surfaces, making it suitable for bioconjugation, the process of linking an analyte to a surface. CDI is frequently employed as a linker in the construction of many biosensor surfaces. Notably, the imidazole functional groups within CDI demonstrate a strong interaction with copper atoms, leading to the formation of charge-transfer complexes [21], [22], [23]. CDI-NF exhibits a large surface area, which is beneficial for biosensing applications as it can efficiently bind to many molecules, making it suitable for detecting and analysing various substances. Additionally, its enhanced catalytic activity makes it an excellent candidate for catalysts, facilitating easier and more efficient chemical reactions with other molecules. Furthermore, CDI-NF demonstrates good magnetic properties, adding to its versatility for potential applications in different fields. Another advantage is its relatively straightforward synthesis process, allowing for the production of large quantities of this nanoflower. Overall, CDI-NF holds great promise as a material with diverse applications, owing to its high surface area, improved catalytic activity, and favourable magnetic properties. Using Laser Scribed

Graphene (LSG) and a hybrid nanoflower made of copper (Cu) and 1,1'-carbonyldiimidazole (CDI), Subramani et al [24] developed a new capacitive aptasensor capable of detecting β-lactoglobulin in milk with a detection limit of 1 attogram per milliliter (ag/ml) .

The early-stage diagnosis and effective treatment of dengue serotype 4 are hindered by the absence of efficient bio-capture materials that specifically target and capture the virus. Current bio-capture materials exhibit insufficient binding affinity towards dengue serotype 4, leading to lower sensitivity and accuracy in diagnosis and treatment. Additionally, many of these materials lack long-term stability and durability, limiting their practical application for dengue serotype 4 capture and detection in real-world scenarios [25]. Furthermore, the intricate and time-consuming synthesis and fabrication methods of these bio-capture materials pose challenges in their large-scale production and practical implementation [26]. Addressing these issues and developing advanced bio-capture materials with high specificity, sensitivity, stability, and scalability is crucial for improving the early detection and management of dengue serotype 4 infections.

In this work, LSG/Mn_3O_4 tetrahedral and carbonyldiimidazole nanoflower was synthesized to create a composite bio-capture material. The composite material was characterized using Transmission Electron Microscopy, Raman Spectroscopy, X-ray photoelectron spectroscopy, Fourier Transform Infrared Spectroscopy and X-ray diffraction, to assess its morphological, structural, and electrical performance. The binding affinity of the composite towards dengue serotype 4 was examined to evaluate its capabilities as a bio-capture material. The potential application of the composite was explored in the context of its suitability and relevance for early-stage diagnosis and efficient treatment of the disease. The bio-characterisation was conducted using Electrochemical Impedance Spectroscopy. By investigating and understanding the properties and performance of this composite material, we

aim to contribute valuable insights into the development of advanced bio-capture materials for disease detection and management.

2.0 Materials and methods

2.1 Materials

The polyimide film was acquired from Avantis Laboratory Supply, and the lignin was isolated from the EFB oil palm [27], The following materials were purchased: ethanol (C_2H_5OH), monoethanolamine, MEA (C_2H_7NO), 1,1′-Carbonyldiimidazole (CDI, reagent grade), copper sulphate pentahydrate, hexamethylenetetramine, HMT ($C_6H_{16}N_2$), manganese (II) nitrate tetrahydrate ($Mn(NO_3)_2{\cdot}4H_2O$), and copper sulphate pentahydrate ($CuSO_4.5H_2O$) were all purchased from Sigma-Aldrich. First Base Chemicals' phosphate buffer saline (PBS, 10 mM, pH 7.4) was used for rinsing, dilution, and as an electrolyte for capacitance tests. The oligonucleotide sequences used were: probe DNA (p-DNA): 5'-amine-C6-C TTC CAC CAG GAG TAC AGC TTC CTC-3'; complementary target DNA (t-DNA): 5' -GAG GAA GCT GTA CTC CTG GTG GAA G- 3'; non-complementary target DNA (nc-DNA): 5'-ATG AAG CTG TAG TCT CAC TGG AAG G-3'; one base mismatched target DNA (m-DNA): 5' -GAG GAA GCT GTA GTC CTG GTG GAA G- 3'; three base mismatched target (tm-DNA): 5' -GAG GAA GCT GTT GAC CTG GTG GAA G- 3' were purchased from Sigma-Aldrich Co., Ltd. (USA).

2.2 Preparation of Laser Scribed Graphene (LSG)

The lignin was coated on the polyimide (PI) film because PI is a good conductor of heat and electricity, which is essential for laser scribing. After cleaning the PI film with ethanol, it was allowed to dry at room temperature. The polyimide substrate covered with lignin was cured for an hour at 50°C before laser scribing. The laser scribing process was chosen because it is

the most straightforward and energy-efficient method of obtaining graphene from graphite. Before the laser scribing process, a computer-aided design of Interdigitated Electrode (IDE) was drawn using the laser scribing machine software. Following laser scribing, the graphene-lignin mixture was separated by dissolving the lignin component in distilled water, leaving the insoluble graphene as a solid residue. The parameters of the laser machine are shown in Table 1.

2.3 Preparation of MnO Seed Solution and Coating of LSG

Manganese acetate dihydrate in ethanol was used to make MnO seed solution (sol gel). monoethanolamine (MEA) was used as a stabiliser. A 0.2 mol/L salt solution is first made and 2.45 g of manganese acetate dihydrate dissolved in 50mL of ethanol. The salt solution is then stirred for 30 minutes on a hot plate at 87℃ and 1000 revolutions per minute. 50.9 µL of the MEA solution was added to the salt solution every 10 minutes for 2 hours. Before use, the mixture is allowed to mature for 48 hours. LSG was then coated with the MnO seed solution. The LSG samples were subsequently placed on the spin coater on a clean commercial polyimide (PI) film with tape to prevent the sample from falling from the vacuum. 2-3 drops of MnO seed solution was added to the samples. The parameters of the spin coater are shown in Table 2. After spin coating, the samples were placed on the hot plate. For the first 20 minutes, the temperature is set to 60℃. The temperature is then raised to 150℃ for 10 minutes. Finally, the temperature was set to 50℃. The solution took approximately 25 minutes to reach the desired temperature.

2.4 Hydrothermal Synthesis

The hydrothermal synthesis method was used to To produce hybrid nanocomposites with improved characteristics by inducing the growth and attachment of nanostructured LSG.

Hexamethylenetetramine (HMT) was used as a reducing agent, and manganese (II) nitrate as a precursor to form manganese oxide nanostructures on the LSG substrate. A solution of HMT and Manganese (II) nitrate was prepared by adding 0.263 g of the HMT solution and 0.471 g of manganese (II) nitrate solution to 75 mL of water in two separate beakers. The beakers were covered with Parafilm and put on a magnetic stirrer for 20 minutes. The manganese (II) nitrate solution and the HMT solution were combined after 20 minutes and placed back on the magnetic stirrer for another 20 minutes. The LSG substrate attached to a Teflon block is then immersed in the solution, ensuring that the entire LSG surface was coveredAluminium foil was used to seal the beaker and placed in an oven for 4 hours. The oven settings are shown in Table 3. The LSG substrate with the formed manganese oxide nanostructures was retrieved after the hydrothermal synthesis process was completed.

2.5 Synthesis of Carbonyldiimidazole-copper Nanoflower (CDI-NF)

CDI-nanoflowers (CDI-NFs) were produced using a modified one-pot synthesis process. A centrifugal tube containing separate CDI solutions (3 ml) in Phosphate Buffered Saline (PBS) at concentrations of 0.1 mg/ml and 0.05 mg/ml was filled with 20 µl of a copper sulphate pentahydrate solution (200 mM). The mixture was vortexed for 30 seconds at 3000 rpm before resting at room temperature for 24 hours, with blue precipitates forming at the bottom of the centrifuge tube. The precipitates were then separated by centrifugation at 4000 rpm for 15 minutes, followed by filtration and rinsed three times with Milli-Q water. The nanofibers were then collected and stored in a refrigerator at -20 °C until further use.

2.6 Material Characterization

Transmission electron microscopy, or TEM (Hitachi, HT7830), was used to analyse the surface morphology and microstructure of the electrode. The electrode's X-ray diffraction and

XRD patterns were obtained at 0.02°/step and 2 s/step using Panalytical X'Pert3 Powder. The studied material's Raman Spectra were obtained using a Horiba, HR800 Raman Spectrometer, which used a 514 nm laser for excitation. The electrode's specific surface area (SSA) and pore diameter distribution were assessed using the Tristar 3020 Plus based on the Barrett Joyner Halenda (BJH) model and the Brunauer-Emmett-Teller (BET) technique. X-ray photoelectron spectroscopy and Thermo Scientific K-X-ray Alpha photoelectron spectroscopy were used to analyse the elemental composition of the electrode. measurements were made using Fourier-transform infrared spectroscopy (FTIR) (Perkin Elmer Spectrum One) to determine the presence of functional groups in the material.

2.7 DNA Immobilization and Hybridization

To facilitate covalent bond interaction with the probe DNA, CDI NF was deposited on the IDE. 5 µL of the probe DNA solution was then added to the IDE, incubated for one hour, and washed with PBS. Target DNA at different concentrations (1 fM to 1 nM) was immobilised on the IDE for hybridization studies. Mismatched and non-complementary DNA sequences were investigated in detail.

3.0 Results and Discussion

3.1 Morphological analysis of CDI-NFs on LSG/Mn_3O_4 Transmission Electron Microscopy (TEM).

The shape and structure of the CDI-NFs on LSG/Mn_3O_4 were examined in further detail using TEM. The homogeneous deposition of Mn_3O_4 is shown in Figures 1 (a-d). The deposition of CDI-NFs on LSG/Mn_3O_4 are shown in Figure 1(b), (c), and (d) with the dark clumps illustrating the distribution of CDI-NFs on the surface, consistent with the 3D flower-like shape that CDI-NFs normally display. More defined complete flower-like morphology was seen

(Fig. 1d) with multiple NFs of ~ 20–30 µm grown in a EIS. The image also clearly shows the close interaction between the black clumps and the LSG/Mn_3O_4. Electrochemical processes and effective electron transport depend on this close contact. CDI-NFs can greatly improve electrochemical performance when added because they have a high surface area and numerous active sites for electrochemical processes. Apart from that, mass transfer and electrolyte diffusion are facilitated by the nanoflowers' porous structure. As a result, the addition of CDI-NF enhances surface area and electrical conductivity.

3.2 Optical Analysis of CDI-NFs on LSG/Mn_3O_4 through Raman Spectroscopy (RAMAN).

Raman was used to determine the structure of a material and the amount of a particular component in a mixture. As shown in figure 2, the smaller peaks around 310 and 350 cm^{-1} is the T_{2g} symmetry mode, which is doubly degenerate and consistent with the tetragonal structure of Mn_3O_4. The sharp peaks around 640 cm^{-1} for both graphs are the distinctive Mn_3O_4 peaks corresponding to the Mn_3O_4 vibrational mode. This distinctive peak is sometimes referred to as the A_{1g} mode, representing the divalent manganese ions' Mn-O breathing vibration in tetrahedral coordination [28]. The peaks observed at 1350, 1577, and 2690 cm^{-1} is attributed to graphene, with the presence of the D band peak at approximately 1350 cm^{-1} closely associated with the level of disorder in graphene [29]. The G and 2D peaks of graphene were identified at around 1577 and 2690 cm^{-1}, respectively [30], [31]. The addition of CDI-NF is correlated with the sample's peak at 2440 cm^{-1}. The peak at 2440 cm^{-1} indicates the presence of CDI molecules around the copper atoms, which is due to the stretching vibration of the carbon-nitrogen double bond (C=N) of the CDI ligand [32]. Moreover, the peak at 2920 cm^{-1}, which indicates the presence of alkyl functional groups, is due to the stretching vibration of the C-H bond of the alkyl groups on the surface of the copper nanoflowers [33]. The water

molecules' OH stretching vibration corresponds to the peak at 3237 cm^{-1}. The existence and structure of water molecules in the sample correspond to this vibrational mode [34].

3.3 Validation of immobilization and hybridization through Fourier-transform infrared (FTIR) spectroscopy X-ray photoelectron spectroscopy (XPS) and X-RAY Diffraction (XRD).

FTIR is an effective analytical technique that assesses a sample's infrared light absorption, transmission, and reflection. The FTIR spectra analysis of the composite material consisting of CDI-NF and Mn_3O_4 reveals distinctive peaks corresponding to various functional groups. In particular, the spectrum displays prominent peaks at 34709 cm^{-1}, attributed to the O-H stretching vibration of water molecules [35]. This finding implies that the CDI-NF surface contains hydrophilic moieties, indicating that the material has a natural ability to bind water. Furthermore, the peaks at 1650 cm^{-1} indicate the presence of C=O stretching vibration of carboxyl and carbonyl functional groups [36]. A wider peak is observed in the bare Mn_3O_4 graph, compared to the peaks with CDI-NF. This variance in peak width can be attributed to the interaction of Mn_3O_4 with CDI-NF, which may lead to changes in the chemical environment and bonding of the Mn-O units, resulting in narrower peaks in the FTIR spectrum.

The peak at 1384 cm^{-1} in Figure 3 could be attributed to continued CH_3 or CH_2 vibrations [37]. According to Pham et al [12], the peak at 1384 cm^{-1} corresponds with carbon-hydrogen (C-H) stretching bonds and illustrates the dynamic behaviour of C-H bonds during stretching vibrations. The amount of C-H bonds in the sample usually corresponds to the amplitude of the C-H peak. An increased concentration of aliphatic hydrocarbons is indicated by a stronger peak. The peak at 1252 cm^{-1} could indicate the continuation of C-O vibrations, possibly related to the P-O stretching in the composite material [38]. When CDI-NF is added, an FTIR spectrum shows a P-O signal at 1252 cm^{-1}, which suggests the sample contains

phosphate groups. The proportion of phosphate groups in the sample is reflected in the peak's intensity. The peak at around 500 cm^{-1} shows the presence of manganese oxide in the sample. The peak at 500 cm^{-1} corresponds to a strong Mn-O bond with the Mn-O-Mn peak at 580 cm^{-1} indicative of the existence of a bridging oxygen atom inside the manganese oxide structure. A Mn-O-Mn bond is created when the bridging oxygen atom joins two manganese atoms. This bond is usually stronger than the Mn-O bond.

The sample's XPS result at 1225 eV is due to the presence of manganese oxide, more precisely Mn_3O_4. XPS radiation can excite the 3p1/2 orbital of manganese. According to IIton et al. (2016),[39] the binding energy of manganese oxide is consistent with the binding energy of the Mn 3p1/2 electron, which is around 1225 eV. Multiple studies have found that the peak at 975 eV is generally connected with the binding energy of the copper (Cu) 2p3/2 orbital. The 2p spectra are complex due to overlapping binding energies for Cu metal and Cu (I) species, as well as shake-up structures for Cu (II) species, with the XPS peak at 975 eV due to the copper 2p3/2 orbital binding energy [40].

Additionally, the binding energy values at 530 eV that were observed are consistent with the predicted oxygen peaks and are compatible with the presence of oxygen in the sample. Carbon's chemical states including sp2 C-atoms and C-N bonds, can be linked to the XPS peak at 653 eV, based on the work by Author [41] who used XPS analysis to show that carbon was present in various chemical states in their investigation of Cu, N-codoped hierarchical porous carbon (Cu-N-C) with a high pyridinic N content. The existence of carbon and nitrogen in the sample is confirmed by the component at 286.3 eV, which corresponds to C-N bonds [42]. The existence of carbonyldiimidazole copper nanoflowers (CDI-NF) and manganese oxide (Mn_3O_4) is suggestive of the presence of Mn, Cu and O. Organic compounds like CDI have been determined to be present based on the existence of C and N.

The XRD spectra of LSG/Mn_3O_4 with and without CDI-NF added are compared in Figure 5. The peaks at 29.1°, 33.08°, 36.3°, 45.8°, 52.3°, 59.1°, 60.8° and 65.3° suggest the presence of Mn_3O_4 [43], which is supported by the tetragonal hausmannite structure of Mn_3O_4 based on XRD analysis. The minor peak at around 74° is indicative of the crystalline planes of Cu , which are not visible in Figure 5 for Day 1, 3 and 5. The CDI-NF causes the LSG/Mn_3O_4 to crystallise more quickly and into smaller crystals, which causes the inclination in graphs (b), (c), and (d) and the subsequent decline. More biomolecules can be bonded to the electrode on a greater surface area, increasing the biosensor's sensitivity [44]. Bare LSG/Mn_3O_4 exhibits a greater degree of crystallinity compared to LSG/Mn_3O_4 with CDI- NF as the XRD pattern of the bare sample shows sharper peaks and greater intensity diffraction peaks. When CDI-NF is added, the Mn_3O_4's crystal structure can get disrupted, resulting in larger peaks and reduced intensity in the XRD pattern.

The electrochemical and catalytic characteristics of Mn_3O_4 are mostly determined by its crystallographic orientation and phase purity. As the plane with the maximum atomic density and reactivity, the (220) plane, shown by the peak at 29.1°, is essential for catalytic activity and electrochemical performance [45]. Similarly, Mn_3O_4-based materials' electrochemical behaviour is influenced by the (220) and (111) planes, which correspond to the peaks at 29.1° and 33.08°. These planes are crucial for charge transfer and redox processes [46][47]. The peaks at 32.5° and 36.3° represent the (222) and (400) planes, which are similarly important for the exposure of active sites and the improvement of catalytic activity in a variety of applications [48].

3.6 ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS): Measurement of Impedance for Surface Functionalization

EIS was used before target detection to evaluate the impedance spectra and the prescribed surface functionalization. EIS applies a small amplitude, alternating current (AC) signal to the biosensor electrode over a wide range of frequencies. The impedance of the system, which is the opposition to the flow of this AC current, is measured. Data Analysis where the measured impedance data is analyzed to extract key parameters, such as Charge Transfer Resistance (R_{ct}). This represents the resistance to electron transfer at the electrode-electrolyte interface. A decrease in R_{ct} often indicates successful binding of the target analyte to the biorecognition element (e.g., enzyme, antibody) on the sensor surface. Then, the Double-Layer Capacitance (Cdl) which reflects the capacitance of the electrical double layer formed at the electrode surface. Changes in Cdl can provide information about the surface area available for electron transfer and the extent of analyte binding. Warburg Impedance where the component arises from the diffusion of electroactive species in the solution. Changes in Warburg impedance can indicate changes in the diffusion rate of the analyte or the redox probe [49]. EIS can detect very small changes in interfacial properties, making it highly sensitive to subtle changes in the sensor environment upon analyte binding. EIS can be used for label-free detection, eliminating the need for additional labeling steps that can complicate the sensing process. EIS can be used to monitor the binding kinetics of the analyte in real-time, providing valuable information about the interaction between the analyte and the biorecognition element. EIS can provide insights into the underlying mechanisms of the biosensing process, such as the rate-limiting steps and the influence of various factors on sensor performance [50], [51]. Three important characteristics from Randles' equivalent circuit were analysed: CPE (constant phase element), Ra (bulk solution resistance), and Rct (charge transfer resistance). The Nyquist plot, depicted in Figure 6, visually represents the semicircles corresponding to the charge transfer resistance (Rct). The variations in each semicircle diameter in Figure 6 (a) represent the modifications made to the changed electrode surface's electrical characteristics at each immobilization stage. The bare electrode has a high impedance value of 79441Ω, indicative of a less conductive surface. Following the addition of CDI-NF,

the impedance decreased to 19776 Ω, potentially due to a modification in the surface properties by CDI-NF immobilization. The introduction of the probe resulted in a further reduction in impedance to 1133 Ω, indicating increased surface conductivity, likely due to successful probe immobilization. The trend shifted when the target was dropped onto the modified electrode, causing an increase in impedance to 11878 Ω.

This change signifies alterations in the electrical properties of the surface, potentially indicative of interactions between the target and the immobilized probe. The charge transfer resistance for the probe is lower than that of bare and CDI-NF due to the large electrostatic charge caused by the bases and phosphorus present in single-strand DNA (probe). As shown in the figure 6 (a), this charge eventually neutralises with additional target with double helix DNA, leading to in an increase in charge transfer resistance. This is further supported by the fact that greater target concentrations from 1f to 1n result in increased charge transfer resistance in figure 6 (b), which is caused by a high number of double helices forming on the sensor's surface and high charge transfer DNA. The observed trend in omega values during each immobilization step provides valuable insights into the success of surface modifications and the dynamic changes in impedance throughout the biosensing process.

Given that the surface functionalization was successful, the limit of detection was further examined by applying varying DENV-4 concentrations (1 fM – 1 nM) to the probe-modified electrode. The analysis involved measuring the electrode using EIS and observing the changes in charge transfer resistance (Rct) and the diameter of semicircles on the Nyquist plot. Based on Figure 6 (b), when the concentration of DENV-4 on the modified electrode increased, the charge transfer resistance and the semicircle's diameter increased. The measured impedance values showed a clear trend: the maximum impedance value of 11878 Ω was correlated with a DENV-4 concentration of 1nm, while the lowest impedance value of 2290 Ω was associated with a concentration of 1fm. The impedance values for 10 pM, 1 pM, and 10 fM are 10858 Ω,

9357 Ω and 5097 Ω respectively. As the concentration increases, the Rct values increase dramatically. This is due to various reasons, including the presence of DENV-4, which increases the number of binding sites on the electrode surface. The larger number of binding sites on the electrode surface increases DENV-4 surface coverage and related interactions leading to a rise in charge transfer resistance.

To investigate the sensitivity of the CDI-Cu-decorated LSG/Mn_3O_4 biosensor, a linear correlation with the difference in the Rct was plotted according to the equation:

$$\Delta Rct = Rct_{hybridization} - Rct_{immobilization}$$

corresponding to the logarithm of the complementary DNA concentrations. As shown in Figure 6 (c), ΔRct increased linearly as the complementary DNA concentration increased. The differences in the values (ΔRct) between the Rct of the immobilized and hybridized species were found to fit the natural logarithm of the target DNA concentrations according to the linear relation: $\Delta Rct = 7.1313 \times 10^{-4} + 4.053 \times 10^{-3}$ with a regression coefficient (R^2) of 0.97373.

This analysis aimed to evaluate how various target sequences affected the charge transfer resistance (Rct) in biosensing. Specifically, the third base mismatch in a DENV-4 target sequence, a non-complimentary target, and a complementary target was investigated as shown in figure 6 (d). There was a clear pattern shown by the Rct values for these. With a Rct value of 11878 Ω, the target sequence unique to DENV-4 showed the strongest reaction to the intended target. The target sequence that had a single base mismatch, on the other hand, had a lower Rct value of 2938 Ω, indicating that the system could identify differences in the target sequence. With a Rct value of 994 Ω, the non-complementary target sequence showed the lowest level of interaction. The biosensing system's ability to discriminate between a complimentary target, a third base mismatch, and a non-complementary target is demonstrated by this observed pattern, which highlights the system's specificity. Morevoer, the consistency of LSG/Mn_3O_4 decorated CDI-NF bioelectrode analysis was evaluated by examining five

samples prepared under identical conditions from the same batch. Figure 6 (e) demonstrates the repeatability curve for this bioelectrode, illustrating a relative standard deviation (RSD) of 3% from five parallel measurements under consistent processing conditions. Furthermore, the bio-electrode's stability was assessed through an 8-week shelf-life study at 2 °C, with weekly measurements. The results indicate relative stability, with only a 14% decrease in activity over the entire 8-week period as shown in figure 6 (f). The results highlight how sensitive the biosensor is to changes in the target sequences, which is essential for precise and targeted detection in biosensing applications.

4.0 Conclusion

A novel composite bio-capture material comprising of Mn_3O_4 and carbonyldiimidazole-nanoflower (CDI-NF) with remarkable performance for the detection of Dengue Virus Type 4 (DENV-4) was successfully synthesized. The material characterization revealed a well-defined structure and composition, which contributed to its enhanced surface functionalization and sensitivity towards DENV-4. The biosensor exhibited high specificity, distinguishing between complementary, mismatched, and non-complementary target sequences, making it a promising tool for DENV-4 detection. The successful development of this composite bio-capture material and the demonstration of its promising biosensing capabilities represent a significant advancement in disease detection.

Future work include further optimizing the synthesis parameters of the composite material. Investigating variables such as laser scribing parameters, sol-gel concentrations, and hydrothermal synthesis conditions could lead to refined material properties and improved performance. It is crucial to validate the biosensor's capabilities in clinical settings by collaborating with healthcare professionals and institutions. Assessing its efficacy in detecting Dengue Virus Type 4 in real patient samples would provide valuable insights into its practical utility. Additionally, exploring multisensor integration is recommended to enhance overall

sensitivity and specificity. Combining the biosensor with other sensor technologies could result in a more comprehensive diagnostic tool. Finally, the biosensor could be modified to detect a broader range of pathogens or analytes. Investigating its adaptability for diagnosing various diseases could contribute to the development of a versatile and multipurpose diagnostic platform.

Credit authorship contribution statement

Jeysree Chelvaraj: Conceptualization, Methodology, Writing - original draft. Sivainesh Devi Remesh: Writing, editing. Saravanan Karupannan: Supervision, Project administration. Veeradasan Perumal: Project administration, Supervision, Conceptualization, Methodology. Mark Ovinis: Visualization, Validation. Subash Gopinath: Formal analysis, Validation. Natarajan Arumugam: Writing - review & editing, Validation. Raju Suresh Kumar: Writing - review & editing, Validation.

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Figure legends

Figure 1: (a) LSG/Mn_3O_4 (b) LSG/Mn_3O_4 with CDI-NF Day 1 (c) LSG/Mn_3O_4 with CDI-NF Day 3 (d) LSG/Mn_3O_4 with CDI-NF Day 5.

Figure 2: Raman Spectroscopy of LSG/Mn_3O_4 and LSG/Mn_3O_4 with CDI-NF.

Figure 3: FTIR spectrum of LSG/Mn_3O_4 and LSG/Mn_3O_4 with CDI-NF Day 1, 3 and 5.

Figure 4: XPS Spectrum of comparison of LSG/Mn_3O_4 with and without CDI-NF.

Figure 5: XRD spectra of LSG/Mn_3O_4 and LSG/Mn_3O_4 with CDI-NF Day 1, 3 and 5.

Figure 6: Impedance spectrum for each step of surface immobilisation.

Table Captions

Table 1 shows the laser scribing required values.

Table 2 shows the spin coating required values.

Table 3 shows the parameters for the oven for hydrothermal synthesis.

Table 4 shows the parameters of the electrochemical impedance spectroscopy test.