Lignin Derived Nanoparticle Intercalation on Nitrogen-Doped Graphene Quantum Dots for Electrochemical Sensing of Cardiac Biomarker

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ABSTRACT

Lignin-scribed graphene (LSG) conjugated with nitrogen-doped graphene quantum dots (N-GQDs) and lignin-derived silver nanoparticles (Ag NPs) was developed through a hydrothermal process for the electrochemical sensing of Troponin I, a cardiac biomarker for Acute Myocardial Infarction (AMI). A nanocomposite with optimal conduction mechanism was developed by varying the N-GQDs doped amount intercalated on the surface of LSG. The nanocomposite was characterised by morphological, physical, and structural examinations. The Ag NPs and N-GQDs were found uniformly distributed on the LSG surface, with selective capture of the biotinylated aptamer probe on the bio-electrode indicative of the specific interaction with Troponin I, resulting in an increment in the charge transfer resistance following hybridisation analysis. The detection limit, as determined through impedance spectroscopy, was 1 fM or 30 fg/mL, with high levels of linearity, selectivity, repeatability, and stability of the sensor. This nanocomposite opens a new avenue for array-based medical diagnostics.

Keywords: lignin-derived graphene; nitrogen-doped graphene quantum dots; silver nanoparticles; troponin I; biosensor

1.0 Introduction

A heart attack, or an Acute Myocardial Infarction (AMI), occurs when there is a disruption of blood flow to the heart [1]. Troponin I, a protein found in human blood is usually released when the heart muscle is injured or damaged following a heart attack. The concentration of troponin I in the blood depends entirely on the injury level of the heart muscles [2]. It is a biomarker for detecting heart injury in patients because it is specific for myocardial injury. Therefore, the development of a technique for accurate, fast, reliable detection of troponin I is of great importance. The electrochemical biosensor method has received much attention and is the most promising approach due to its high accuracy, sensitivity, ease of use, fast response, and low cost [3,4].

The three main components in a biosensor are the active material/substrate, the transducer and the bioreceptor-analyte/ probe-target [5]. The most common active material used is multiwall carbon nanotubes (MWCNT), silica nanoparticles, carbon nitride tube, and graphene [6,7]. Graphene is a two-dimensional carbon nanostructure with extraordinary physical and chemical properties. However, the traditional synthesis of graphene via chemical vapour deposition (CVD), chemical or physical exfoliation is very tedious, requires a lot of energy, and high temperatures, is time-consuming, uses toxic chemicals, and cannot be scaled [8]. Furthermore, graphene is made from non-renewable fossil fuel, usually coal. The depletion of

fossil fuels reduces the future availability of these fuels. Thus, an alternative resource is much needed for graphene synthesis.

In that regard, oil palm empty fruit bunch waste lignin can be converted into graphene through the visible carbon dioxide laser scribed method. Lignin biopolymer is a sustainable, biodegradable and low-cost natural resource [9]. It is a natural reducing agent, making it an attractive green polymer material for biosensing applications [10]. Researchers have reported studies on kraft lignin and organosolv lignin for biosensor applications [11]. A high-heat laser-scribed method with optimised parameters effectively converts the lignin into three-dimensional sp^2 carbon atoms (laser-scribed graphene) [12]. The laser-scribed method does not need binders, post-treatment or chemical infusion and rapidly produces graphene on a large scale in a short time with excellent productivity and reproducibility. Compared to conventionally synthesised graphene, laser scribed graphene (LSG) has a large surface area, high porosity, biodegradability, biocompatibility, and is environmentally friendly, and renewable. However, LSG has weak electroconductivity due to the amorphous nature of lignin [13].

Graphene quantum dots (GQDs) are zero-dimensional carbon nanostructures with microscopic dimensions and a single atomic layer of nano-sized graphite [14] with nitrogen-doped graphene quantum dots (N-GQDs) having enhanced intrinsic properties, more active sites and higher electrical characteristics than GQDs. N-GQDs were chosen to augment the electroconductivity of LSG due to the amorphous nature of LSG, which allows it to maintain its inherent electrical properties. N-GQDs can improve the kinetics of electron transfer, as nitrogen modifies the electronic configuration of GQDs [15]. Furthermore, nitrogen doping in GQDs introduces nitrogen-containing functional groups, such as amide groups, on the surface, leading to specific chemical interactions. N-GQDs possess a quantum-sized structure with a large surface area, which enables large density of functional groups to be deposited on its surface, especially during layer-by-layer surface modification through the chemical process [16], allowing for effective interactions between aptamer and troponin I. Furthermore, N-GQDs improves chemical binding to anchor nanoparticles, enhancing electrocatalytic activity and stability. N-GQDs have natural directing and reducing properties, although they have limited solubility [17].

On the other hand, noble metals such as silver nanoparticles (Ag NPs) are extensively used for high-performance sensing due to their excellent catalytic properties in redox reactions, intrinsic peroxidase properties, conductivity, large surface area, and biocompatibility [18,19]. Silver, which has identical bulk lattice constants to gold, is relatively inexpensive. However, strong van der Waals force between Ag NPs causes severe aggregation and instability,

resulting in significant loss in accessible electrochemical activity and detection sensitivity [20]. To reduce the aggregation of nanoparticles, Ag NPs are generally conjugated with organic/inorganic material to prevent agglomeration and improve their electrocatalytic activity and stability. In addition, the ease of oxidation of Ag NPs surface is an intrinsic disadvantage, which will disrupt its catalytic performance due to the blocked surface-active area. Furthermore, the usual synthesis method of Ag NPs uses harmful chemicals that need high-energy consumption. Therefore, a lignin-based Ag NPs synthesis method was introduced in this study.

In this work, N-GQDs and green Ag NPs were intercalated with LSG to develop an electrochemical troponin I biosensor. The synergistic LSG N-GQDs Ag NPs nanocomposite overcomes the limitation of individual materials with enhanced electrical, physical, structural and mechanical properties. Furthermore, stabilising N-GQDs through $\pi - \pi$ stacking interaction on LSG and conjugation of Ag NPs around LSG_N-GQDs improved the sensitivity, selectivity and stability of the developed biosensor. N-GQDs and (rod-shaped) troponin I, are in the nanometer range, facilitating their complex formation. The basic chemistry with the biomolecular assembly is shown in Figure 1. Troponin I level in normal healthy human blood is below 0.04 ng/ml and above 0.40 ng/ml after an AMI symptom, well aligned with the concentration range and limit of detection of the current study [21,22].The present method is also suitable for the physiological ranges. This low-cost and high-performance electrochemical biosensor is promising for clinical diagnosis.

2.0 Experimental

2.1 Materials and Reagents

Lignin extracted from oil palm's empty fruit bunch was purified [12] for conversion to graphene. Chemicals such as 16-Mercaptohexadecanoic acid, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Silver Nitrate ($AgNO_3$), N-hydroxysuccinimide (NHS), Streptavidin, Phosphate Buffered Saline (PBS), and (3-Aminopropyl) triethoxysilane (APTES) were obtained from Sigma-Aldrich Co., Ltd. (USA), while Potassium Hydroxide (KOH), Ethanolamine, and Ethanol Absolute were obtained from Merck & Co (USA). Citric acid-1-hydrate was purchased from Bendosen, Laboratory Chemicals, while ethylenediamine was obtained from QREC, Grade AR, (Asia) Sdn. Bhd, Malaysia. All these chemicals were used as received without additional purification. The Screen-Printed Carbon Electrode (SPCE) was purchased from Metrohm (Malaysia) Sdn. Bhd. The oligonucleotides were purchased from Avantis Laboratory (USA). A detailed description of the oligonucleotide sequence dilution and

characterisations can be found in the supplementary information. The oligonucleotide sequences utilised in this current work are as follows:

Probe (Aptamer) – 5′ – CGT GCA GTA CGC CAA CCT TTC TCA TGC GCT GCC CCT CTT AAA AAA AAA AAA AAA AAA AAA A-3′; Biotin oligo linker – 5′ - / 5Biosg // iC6Sp / TTT TTT TTT TTT TTT TTT TT −3′; Target – Troponin I; Control – Troponin T; Human Serum of male AB plasma were purchased from Sigma-Aldrich Co., Ltd. (USA).

2.2 Fabrication of LSG

Lignin-derived graphene was produced via the laser scribing method [23]. Initially, 20% of the lignin solution was prepared by adding 10 grams of lignin powder in 50 ml of distilled water and stirred until a homogenous lignin solution formed. The homogenous lignin solution was coated on a glass substrate and left to dry for 30 minutes in an oven at 50 °C. The dried lignin glass substrate was then inserted in a laser-scribed machine under optimised parameters of 70% laser power, 50% laser speed, and 500% pulse per inch. A CO_2 laser was used to penetrate a lignin-coated glass substrate forming graphene after 30 minutes. and the graphene was placed in a desiccator to avoid contamination.

2.3 Preparation of N-GQDs

N-GQDs were prepared by a method reported elsewhere [24], with minor modifications. Briefly, the hydrothermal method was conducted by diluting citric acid (CA) and ethylenediamine (EDA) as carbon and nitrogen precursors, respectively. The detailed description of the fabrication is given in supplementary information. The by-product of N-GQDs was further diluted into 50, 100, 500 and 1000 ppm.

2.4 Synthesis of green Ag NPs

Green Ag NPs were synthesised by following the experimental procedure reported by [12]. In brief, a 1000 ppm silver nitrate (Ag NO_3) solution was added gradually to 1000 ppm of lignin solution under constant stirring conditions until the homogenous solution changed from dark brown to brownish black, which confirms the reduction of the silver ions to Ag NPs, aided by the lignin. The surface plasmon excitation of Ag NPs causes the colour changes of the solution. This shows that aqueous silver ions could be reduced by the lignin extract to yield stable Ag NPs in water.

2.5 Fabrication of LSG_N-GQDs_Ag NPs nanocomposites

The LSG_N-GQDs_Ag NPs nanocomposites were prepared by the method reported elsewhere [25,26] with minor changes. In brief, 50 mg of LSG was diluted in 30 ml of distilled

water and magnetically stirred for 30 minutes in a beaker. Then, 15 ml of 1000 ppm of Ag NPs and 15 ml of 50 ppm of N-GQDs were added to the same beaker and stirred for 10 minutes. The mixture was then transferred to a Teflon-lined stainless-steel autoclave and heated at a temperature of 180 °C for 9 h. During the thermal treatment, the N-GQDs and Ag NPs were attached and intercalated with LSG through $\pi - \pi$ stacking interaction. After heating, the autoclave was left to cool for a few hours. The resulting product, a solid black-coloured, was collected via centrifugation and washed three times with distilled water. Finally, the nanocomposites were dried for 12 hours at 60 °C. The same procedure was repeated by replacing 50 ppm of N-GQDs with 100, 500, and 1000 ppm. The corresponding nanocomposites were named LSG_N-GQDs_Ag NPs-50, LSG_N-GQDs_Ag NPs-100, LSG_N-GQDs_Ag NPs-500 and LSG_N-GQDs_Ag NPs-1000

2.6 Immobilisation and hybridisation on modified SPCE

Chemical modification on SPCE was performed for aptasensor fabrication. Initially, 10 µL of potassium hydroxide (1 M, pH 9.2) was dropped on the SPCE carbon surface and incubated for 5 minutes. Next, the SPCE/KOH surface was incubated with 10 µL of 2% APTES for an hour to activate the amine surface, followed by incubation with 10 µL of LSG_N-GQDs_Ag NPs nanocomposite for 30 minutes. A proportion of 1 mg in 1 ml was then used to dilute functionalised LSG_N-GQDs_Ag NPs nanocomposite. Next, 10 µL of a complex mixture (16-mercaptohexadecanoic acid, NHS, and EDC) was dropped and incubated for an hour to form a stable side reaction with a free functional group for the detection of proteins, followed by 10 µL of streptavidin with 30 minutes incubation time. To cap the non-specific interaction sites, 10 µL of ethanolamine was dropped and incubated for 30 minutes. Finally, 10 µL of biotinylated aptamer was dropped and incubated for 15 minutes. The biotinylated aptamer was prepared by diluting 2.5 µL of aptamer and biotinylated oligo linker in separate 47.5 µL of pure water. After that, both mixtures were combined to form 1 µM of biotinylated aptamer, which was annealed at 60 °C for 1 minute and cooled down at room temperature. The additional oligos in the linker act as a spacer. A buffer solution of 10 mM PBS at pH 7.4 was used to wash thoroughly after each modification.

Electrochemical impedance spectroscopy (EIS) analysis was conducted to determine the chemical reaction of the assembled molecules on the surface-modified electrode [27,28]. The chemical modification is illustrated in Fig. 1. As for the hybridisation process, Troponin I concentrations ranging from 30 ng/mL - 30 fg/mL were diluted through serial dilutions. Later, 10 µL of all diluted Troponin I concentrations were dropped one at a time. Each concentration was incubated for 10 minutes and washed thoroughly with 10 mM PBS of pH 7.4 to remove

the unbonded target. After washing, an impedance analysis was performed. The complete hybridised aptasensor was stored at 4 °C when not in use.

The same hybridisation procedure was done with human serum, using a dilution factor of 1:10 000, to determine the hybridisation capability of the developed biosensor for an actual sample. The stability of the hybridised electrode was determined every week by dropping 10 µL of buffer solution on the surface of the electrode. The repeatability of the electrode was examined by taking measurements using different electrodes from the same batch, for results comparison of every electrode when the aptamer was immobilised on SPCE.

In short, a fixed amount (10 µl) of aptamer was dropped on the surface-modified SPCE at an optimised concentration of 1 µM. PBS, a buffer with a nearly neutral pH, was used to vary the troponin I concentration through serial dilution for repeatability. The PBS solution was used to wash the excess biomolecules after 10 minutes of incubation for each troponin I concentration added to the modified SPCE surface. The diluted biomolecules are stored in a freezer when not in use. The biomolecule detection was done under low temperature. Similar optimum levels were used with human serum analysis.

3.0 Results and discussion

EIS was conducted to assess the optimum and conduction mechanism of LSG_N-GQDs_Ag NPs nanocomposites at various N-GQDs doped levels. The Nyquist plots of LSG_N-GQDs_Ag NPs nanocomposites at different doped levels of N-GQDs: 50, 100, 500, and 1000 ppm are depicted in supplementary Fig. S1. The semi-circle of the Nyquist plot, related to the charge transfer resistance, shows that the LSG_N-GQDs_Ag NPs nanocomposites with 50 ppm of N-GQDs have a larger diameter than the LSG_N-GQDs_Ag NPs nanocomposites with 1000 ppm of N-GQDs. The impedance and dielectric constant decreases as the amount of N-GQDs in LSG_Ag NPs increases, which directly affects the conductivity of nanocomposites. This variation is attributed to the attachment of conductive N-GQDs on the surface of LSG_Ag NPs nanocomposite. The N-doping could induce the enhanced atomic charge density and asymmetry into the spin density on LSG structure, which directly promotes the charge transfer from N-GQDs to the supported Ag NPs, resulting in an excellent interfacial electron transfer of nanocomposites. The LSG_N-GQDs_Ag NPs with the addition of 1000 ppm of N-GQDs were therefore selected for this study as it has the lowest charge transfer resistance and improved electron transfer performance. The bi-functional LSG_N-GQDs_Ag NPs nanocomposite offers unprecedented nanostructures, high catalytic activity, improved sensitivity, and stability for biosensing applications. The intrinsic morphological and structural properties of LSG_N-GQDs_Ag NPs nanocomposites were subsequently investigated in this study. The selective bio-capture of biotinylated aptamer and hybridisation with Troponin I is

illustrated in Fig. 1. To elucidate the biosensing capability of this novel nanocomposite, the biomolecular interaction was examined using impedance spectroscopy.

3.1 Field-Emission Scanning Electron Microscopy (FESEM)

The surface morphology and energy dispersive x-ray spectroscopy (EDX) of LSG_N-GQDs_Ag NPs nanocomposites at different doped amounts of N-GQDs were characterised through FESEM and shown in Fig. 2 (a-l). The FESEM and EDX images of Fig. 2(a-c), represent the LSG_N-GQDs_Ag NPs-50 nanocomposites, Fig. 2(d-f) represents the LSG_N-GQDs_Ag NPs-100 nanocomposites, Fig. 2(g-i) represents the LSG_N-GQDs_Ag NPs-500 nanocomposites and Fig. 2(j-l) represents the LSG_N-GQDs_Ag NPs-1000 nanocomposite. The surface of the synthesised nanocomposites appears thicker, rougher, and is irregular in shape and size, as shown in Fig. 2 (a,d,g&i) at low magnification, compared to the bare structure of LSG, N-GQDs, and Ag NPs displayed in Fig. S2. The surface morphology in the form of wrinkled sheets, clumps, and flakes were also discovered, which is attributed to the thermal attachment of lignin-derived graphene and N-GQDs, forming a strong intercalated bonded structure. The spherical shape of the N-GQDs structure was found in Fig 2e, attributed to a higher doped amount of N-GQDs during the synthesis of a nanocomposite. The magnified FESEM images in Fig. 2(b,e,h,&k), revealed the presence of minute spherical Ag NPs attached to the surface of the LSG_N-GQDs structure. Although no reducing agent and surfactant were used during the synthesis, lignin-derived Ag NPs with uniform particles size can be produced through a hydrothermal process with the presence of LSG and N-GQDs. The diameter of Ag NPs on LSG_NGQDs ranged between 0.2 to 0.6 nm with the spherical Ag NPs evenly distributed and not stacked onto one another. Also, the nanoparticles have not been aggregated during the hydrothermal process. The EDX spectrum of the fabricated LSG_N-GQDs_Ag NPs nanocomposites is presented in Fig. 2(c,f,i,&l). The EDX spectrum demonstrates the presence of carbon (C), nitrogen (N), oxygen (O), and silver (Ag) peaks in all LSG_N-GQDs_Ag NPs nanocomposites. The EDX results prove that as the doped amount of N-GQDs increases, the weight percentage of C, and N elements also rises. These results confirm the deposition of N-GQDs and Ag NPs on LSG through the hydrothermal process. Impurities were also present due to the lignin residues. To justify the EDX findings, elemental mapping of LSG_N-GQDs_Ag NPs-1000 nanocomposite was studied (Fig. S3), which was consistent with the FESEM images, revealing a uniform presence and distribution of oxygen and carbon from LSG, nitrogen from N-GQDs and silver in the structure of the prepared nanocomposites.

3.2 Transmission Electron Microscopy (TEM)

The magnified morphology of the LSG_N-GQDs_Ag NPs-1000 nanocomposite was further confirmed by TEM. The TEM images at both low and high magnifications of the fabricated LSG_N-GQDs_Ag NPs-1000 nanocomposite are shown in supplementary Fig. S4. The low-magnified TEM image in Fig. S4a shows that LSG, N-GQDs, and Ag NPs were attached and are dense at the selected LSG surface. The dark, irregular, oval-shaped structure of N-GQDs was observed and scattered around the LSG surface. The magnified TEM image of Fig. S4b revealed the intercalation and attachment of N-GQDs with LSG. The average length of the spotted N-GQDs is approximately 150 nm to 300 nm, and the diameter is around ~ 100 nm. Deposition of Ag NPs onto LSG and at the edges of N-GQDs are seen in higher magnification TEM image (Fig. S4c) as dark, spherical, irregularly shaped nanostructure. The diameter of Ag NPs is between 5 nm to 30 nm. Uniformly dispersed and small-sized Ag NPs are highly desirable for optimal electrochemical performance. The coupling of LSG and N-GQDs served as anchor and nucleation sites for the growth of Ag NPs and effectively prevented Ag NPs from agglomeration.

3.3 X-ray Diffraction (XRD)

An XRD analysis was performed to investigate the plane orientation and crystallinity of the fabricated LSG_N-GQDs_Ag NPs nanocomposites (Fig. 3 (d-g)). The results were compared with bare LSG (Fig. 3a), N-GQDs (Fig. 3b) and Ag NPs (Fig. 3c). The diffraction peaks observed in LSG were at 18.8°, 21.57°, 23.4°(0 0 2), 25.41°, 32.19°(0 0 2), 34.29°(1 0 0), 45.59°(2 0 0) and 47.69° (1 0 2), denoting the coexistence of reduced graphene oxide (rGO), graphene oxide (GO) and lignin residues due to incomplete laser scribing process [12,29]. A peak at 18.8° indicated the presence of incomplete oxidation [30]. Well-defined diffraction peaks at 23.4°, corresponding to the (0 0 2) plane, suggests good interlayer distance of graphene and oxygen reduction [31,32]. The presence of the (1 0 2) plane peak at 47.69° confirmed the structure of rGO [30]. The absence of a clear peak at (0 0 1) plane indicates that a significant amount of oxygen was removed during laser scribing. The XRD pattern of N-GQDs displayed a broad peak at around 23°, characteristic of the (0 0 2) diffraction [33]. The oxidation degree of N-GQDs determines the interlayer spacing, influenced by various functional groups dispersed on the basal plane. As for the Ag NPs XRD pattern, sharp peaks at 38.3° (1 1 1) and 44.1° (2 0 0), indicates a defect-free and high crystallinity structure. Short peaks at 64.3° (2 2 0) and 77.1° (3 1 1) corresponds to crystal planes of Ag nanoparticles. These Ag NPs peaks are in good agreement with the face-centred cubic structure of Ag nanocrystals (JCPDS04-0783) [34,35]. The XRD peaks of the synthesised LSG_N-GQDs_Ag NPs nanocomposites exhibited a slight shift in the angle of appearance and changes in intensity. The peaks that appeared at around 38.75°, 44.27°, 64.57°, and 77.44° are attributed to the (1 1 1), (2 0 0), (2 2 0), and (3 1 1) crystal planes of Ag nanoparticles respectively. The

high intensity of these peaks caused the suppression and invisibility of some other peaks, leading to overlapping due to the proximity of the areas where the peaks appear. Furthermore, a broader peak at 24.2° (0 0 2) of N-GQDs and short peaks of LSG were observed in all the synthesised LSG N-GQDs Ag NPs nanocomposites. The XRD analysis confirmed the successful blending of LSG, N-GQDs, and Ag NPs due to the oxygen-rich groups on the LSG N-GQDs surface, which can stabilise the deposited Ag NPs.

3.4 Fourier Transform-Infrared (FT-IR) spectroscopy

FT-IR analysis was performed to study the functional group and its vibrational characteristics present on the outermost layer of the fabricated nanomaterials. Fig. 3 (h-n) depicts the FT-IR spectra of LSG (Fig. 3h), N-GQDs (Fig. 3i), Ag NPs (Fig. 3j), and LSG_N-GQDs_Ag NPs nanocomposites at N-GQDs with 50 ppm (Fig. 3k), 100 ppm (Fig. 3l), 500 ppm (Fig. 3m) and 1000 ppm (Fig. 3n) of doping. The major absorption peaks of LSG_N-GQDs_Ag NPs nanocomposites at different doping amounts of N-GQDs are distinct at 3433.5 cm^{-1}, 1722.5 cm^{-1}, 1621.3 cm^{-1}, 1390.8 cm^{-1}, and 1165.4 cm^{-1}, which are assigned of O-H, C=O, C=C, C-N, and C-O groups, respectively. The O-H, C=O, and C=C groups are due to the presence of LSG. The appearance of a peak at 1390.8 cm^{-1} and 1621.3 cm^{-1} implies that the C-N bond and C=N bond are present in the nanocomposite structure, and nitrogen atoms are successfully attached to the structure. A peak at 1722.5 cm^{-1} (C=O bond) in LSG_N-GQDs_Ag NPs nanocomposites confirms the formation of Ag NPs. The broad O-H bond (hydroxyl) at 3433.5 cm^{-1} is similar for all fabricated materials and is caused by the adsorption of water molecules. Therefore, characteristic peaks of different components can be detected in all fabricated nanocomposites, with the carboxylic acid carbonyl groups' peak intensity reduced. At the same time, the peak intensity of the carbon-carbon double bond is increased. The FT-IR analysis of LSG_N-GQDs_Ag NPs nanocomposites has validated the successful pairing of LSG, N-GQDs, and Ag NPs in the nanocomposites.

3.5 Raman Spectroscopy

Raman spectroscopy is used to study the structural order, vibrational energy modes and crystalline size of LSG and LSG_N-GQDs_Ag NPs-1000 nanocomposite, as shown in supplementary Fig. S5. LSG and LSG_N-GQDs_Ag NPs-1000 nanocomposite exhibited two major peaks that are attributed to D band and G band. The D and G bands of LSG appeared at 1353.5 $cm^{-1,}$ and 1585.3 cm^{-1}, whereas for LSG_N-GQDs_Ag NPs-1000 nanocomposite was found at 1355.2 and 1587.4 cm^{-1}. The D band is related to structural defects or lattice distortion and the G band refers to the E_{2g} mode of sp2-bonded carbon atoms of graphene [36]. The G band also refers to the in-plane vibrations of C-C double bonds. The slight peak

shift of the LSG_N-GQDs_Ag NPs-1000 compared to LSG is attributed to phonon stiffening of N-GQDs and change transfer of Ag NPs, indicating a strong interaction between LSG, N-GQDs, and Ag NPs [37,38]. The intensity of the D band and G band was calculated using a formula of I_D/I_G ratio, valued at 0.70 for LSG and 0.85 for LSG_N-GQDs_Ag NPs-1000. The intensity ratio of I_D/I_G is attributed to the degree of functionalisation [39]. The ratio was increased from 0.70 to 0.82 after the addition of functionalised N-GQDs and Ag NPs, indicating the N-GQDs and Ag NPs covalently bonded onto the surface of the LSG through a hydrothermal process. Furthermore, the crystalline size (L_a) of the synthesised materials can be estimated using the Tuinstra-Koeing relation [40] where the λ is equivalent to 514 nm.

$$L_a\ (nm) = (2.4 \times 10^{-10})\ \lambda^4\ (I_D/I_G)$$

The L_a of LSG and LSG_N-GQDs_Ag NPs-1000 nanocomposite was assumed to be 11.73 nm and 14.24 nm respectively. The nanocomposite shows greater crystallinity owing to the surface-enhanced Raman scattering activity of Ag NPs.

3.6 X-ray photoelectron spectroscopy (XPS)

XPS analysis was conducted to examine the elemental, chemical compositions and electronic state of an atom within synthesised nanomaterials, as shown in Fig. 4. The survey spectrum in Fig. 4a shows a clear comparison between LSG, N-GQDs, and LSG_N-GQDs_Ag NPs-1000 nanocomposites. The survey spectrum of the LSG_N-GQDs_Ag NPs-1000 nanocomposite displays four major peaks at 285.2 eV (C1s), 374.2 eV (Ag 3d), 400.5 eV (N1s), and 532.0 eV (O1s), which confirms the successful formation of the nanocomposite. Fig. 4b displays a high-resolution spectrum of carbon, C1s, deconvoluted into five peaks at 284.4 eV, 285.2 eV, 286.6 eV, 288.1 eV, and 289.5 eV attributing to C-C, C-N, C-O, C=O, and O-C=O bonds, respectively [41]. The dominance of the C-N bond suggests that the nanocomposites have N-GQDs in their structure and are well distributed. The shorter peak of carbon-carbon bonds over carbon-oxygen bonds implies less deoxygenation reaction occurred during the hydrothermal process. The N1s spectra in Fig. 4c have been divided into three major peaks at 399.5 eV, 400.5 eV, and 401.8 eV representing pyridinic, pyrrolic, and graphitic nitrogen atoms respectively. Generally, pyridinic functions to occupy the edges or defects of the carbon materials. The pyrrolic in the synthesised nanocomposite has a higher intensity than pyridinic, which is attributed to less defect as most of the nitrogen atoms of the N-GQDs structure are in the pyrrolic form [42]. The graphitic peak implies the substitutional doping of carbon in the hexagonal graphene matric by nitrogen [43]. As for Fig. 4d, an obvious peak of silver was shown at 368.2 eV and 374.2 eV refers to Ag $3d_{5/2}$ and Ag $3d_{3/2}$, suggesting that the Ag were anchored around LSG_N-GQDs surface. The XPS analysis demonstrated

that LSG_N-GQDs_Ag NPs-1000 nanocomposite had been successfully fabricated through the mentioned process.

3.7 Biomolecular interaction analyses by impedance spectroscopy

A commercialised screen-printed carbon electrode (SPCE) was used to develop a biosensor to detect troponin I. SPCEs usually consists of a working, reference and counter electrode printed on a wide range of plastic or ceramic substrates that can be easily modified according to the requirements. The working electrode sensing area comprises a carbon surface area. The layer-by-layer modification for biotinylated aptamer-troponin I interaction on the sensing area of SPCE was analysed via electrochemical impedance spectroscopy (EIS), and the result was generated as a Nyquist plot. The addition of each layer has its own incubation of 15 to 60 minutes. This surface modification was done at room temperature, and the complete hybridised aptasensor was stored at 4°C when not in use.

A precise layer-by-layer modification is necessary for the strong attachment of LSG N-GQDs Ag NPs-1000 nanocomposite on the SPCE chip surface. The SPCE chip was initially treated and modified with KOH and APTES to activate the SPCE surface area of the working electrode [44]. The APTES binds with the -OH group, leaving the amine group unreacted [45]. APTES is a silane that supplies amino acids to functionalise the oxide surface. The high surface energy of the oxide surface rapidly interacts and forms a covalent bond with silane molecules [46]. The unreacted amine group binds with the uniformly distributed silver ions in LSG N-GQDs Ag NPs-1000 nanocomposite and the Ag NPs linked to the NH_2 groups of the APTES covalently causes a charge transfer resistance (Rct) value of 13K Ω, as displayed in the Nyquist plot in Fig. 5a. [47]. The fabricated nanocomposite enhances the charge transfer due to the synergistic effect of the LSG, conductive N-GQDs, and noble metal Ag NPs. Therefore, the energy density and electron transfer efficiency depend entirely on the fabricated nanocomposites.

A complex mixture comprised of 16-mercaptohexadecanoic acid, NHS and EDC was subsequently added on SPCE, boosting the Rct value to 15K Ω. The thiol branch (-SH functional group) of 16-mercaptohexadecanoic acid binds with Ag NPs of LSG N-GQDs Ag NPs-1000 nanocomposite. In contrast, the carboxylic group binds with the amine group of streptavidin, with a Rct value of 22K Ω. The NHS and EDC in the complex mixture form two additional branches of the carboxylic group for additional streptavidin to be attached. Furthermore, streptavidin has four unique active sites, allowing more biotin aptamer attachments [48]. NHS and EDC in the complex mixture act as activators to activate and intensify the surface-modified electrode [48]. Besides that, it is also used to stabilise the interaction between the amine and carboxyl group (streptavidin). Ethanolamine blocks the

unreacted functional groups from the electrode surface that would otherwise compete with reacted functional groups, with the Rct value 25K Ω [28]. The biotinylated aptamer binds with streptavidin, forming a streptavidin-aptamer complex, reaching a Rct value of 27K Ω [49]. The increment in the Rct value of aptamer shows that its immobilisation on streptavidin is a success. Successful capturing of Troponin I by aptamer was also observed, as the Rct value increased to 32K Ω. The increment in the Rct value for each chemical modification depends on the stacking and binding of added materials on the SPCE surface. Therefore, the aptamer-troponin I interaction following the chemical modification of LSG N-GQDs Ag NPs-1000 nanocomposite was successful and will facilitate the detection and monitoring of biomarkers.

Impedimetric spectroscopy was used to further analysed the aptamer-modified LSG_N-GQDs_Ag NPs-1000 electrode for various concentrations of Troponin I to determine the detection limit of the aptamer-modified nanocomposite electrode to capture Troponin I, as shown in Fig. 5b. Troponin I was diluted through serial dilution from 30 ng/mL to 30 fg/mL. Due to the strong interaction of aptamer and Troponin I, the Rct value increases as the Troponin I concentration increases. The resulting Rct confirms the successful detection of a linear range of Troponin I on the aptamer-modified nanocomposite electrode, demonstrating the superiority of the sensing elements. The successful bio-sensing mechanism of the developed LSG_N-GQDs_Ag NPs-1000 electrode was because of the fabricated nanocomposite's larger surface area and electroconductivity properties. Furthermore, as Ag NPs have natural stabilising and reducing properties, no synthetic chemical is required as a stabiliser and reducing agent, thus promoting the biomolecular interaction on the modified electrode. The synergistic effect of LSG, N-GQDs, and Ag NPs improves the charge transfer mechanism, which results in excellent electrochemical performances,

A similar serial dilution under the same experimental condition was prepared utilising human serum. Human serum dilution was prepared and analysed through impedance spectroscopy to investigate the ability of aptamer-modified LSG_N-GQDs_Ag NPs-1000 electrode to hybridise with specific Troponin I nucleic acid with the presence of numerous biomolecules in human serum, as shown in Fig. 5c. The Nyquist plot shows a similar Rct increment trend with real Troponin I concentrations. The Rct value increases as the human serum concentration increases, proving that there is an interaction between the aptamer and Troponin I in human serum and that the developed biosensor has high specificity capability. The performance of the aptamer-modified LSG_N-GQDs_Ag NPs-1000 biosensor was differentiated and compared with previously reported biosensors. The values are given in supplementary Table S1, with the aptamer-modified LSG_N-GQDs_Ag NPs-1000 biosensor more selective than other biosensors.

3.8 Linearity, selectivity, repeatability, and stability of developed biosensor

The analytical performance of LSG_N-GQDs_Ag NPs-1000 bio-electrode was further tested, as shown in Fig. 6(a-d). In brief, the LSG_N-GQDs_Ag NPs-1000 bio-electrode has good linearity, and a detection limit of 1 fM or 30 fg/mL was estimated using a signal-to-noise ratio of more than 3σ. The cross-selectivity with other proteins and control (Troponin T) is also shown in Fig. 6b. The value of the Rct does not vary significantly in the presence of unrelated biomolecules, indicating non-influence of the individual interferants. The Rct of aptamer interaction with Troponin I is three folds larger than immobilisation of aptamer on LSG_N-GQDs_Ag NPs-1000 electrode. The repeatability bar chart of the developed LSG_N-GQDs_Ag NPs-1000 biosensor, as displayed in Fig.6c, has a relative standard deviation (RSD) of 5.4%, with five parallel measurements prepared under similar processing conditions. The stability result (Fig. 6d) revealed that the prepared bioelectrode is very stable and loses only 9% of its activity, retaining 91% stability even after six weeks. Therefore, the developed biosensor possesses the potential for early monitoring of Troponin I in a patient with AMI.

4.0 Conclusion

Nitrogen-graphene quantum dots (N-GQDs) and green silver nanoparticles (Ag NPs) decorated on porous three-dimensional lignin-derived laser scribed graphene (LSG) are potential candidates for developing a highly selective and sensitive troponin I biosensor. N-GQDs and Ag NPs outperform LSG in terms of electroconductivity and aptasensing ability. The variation in synthesised nanocomposite's structural, physical and binding affinity provides large surface-active sites for immobilisation of biotinylated aptamer for troponin I detection. The conjugation of LSG, N-GQDs and Ag NPs overcomes their limitations in terms of electroconductivity (LSG), severe self-stacking (N-GQDs) and nanoparticles agglomeration (Ag NPs). The LSG_N-GQDs_Ag NPs with 1000 ppm of N-GQDs nanocomposite has the best impedance resistivity, with an I_D/I_G ratio of 0.85 and a crystalline size of 14.24 nm. The LSG_N-GQDs_Ag NPs-1000 bio-electrode can detect troponin I in concentrations up to femtomolar with a small sample volume of 10 µL. The LSG_N-GQDs_Ag NPs-1000 biosensor has higher selectivity for troponin I even in the presence of various biomolecules, a stability of 91 % and a RSD of 5.4 % through impedance analysis. This approach may be expanded for high throughput and multiple diagnoses on a single sensing platform.

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Author Contributions

Fabrication and development of nanocomposites were conducted by M.V. Experiments and drafted manuscript were carried out by M.V with the help of S.R, P.B.R, M.N.M.I, S.C.B.G, H.L.L, M.O, S.K, N.A, and R.S.K proof-read the manuscript. V.P supervised the work. All authors analysed the results and contributed to the discussion presented in the manuscript.

Additional Information

The authors declare no competing financial interest

Figure legends

Figure 1: Schematic illustration of synthesised LSG_N-GQDs_Ag NPs nanocomposite on modified surface for biotinylated Aptamer – Troponin I interaction.

Figure 2: FESEM and EDX images of LSG_N-GQDs_Ag NPs nanocomposites. (a) Low magnifications, (b) high magnifications and (c) EDX images of LSG_ N-GQDs_Ag NPs-50; (d) Low magnifications, (e) high magnifications and (f) EDX of LSG_N-GQDs_Ag NPs-100; (g) Low magnifications, (h) high magnifications and (i) EDX of LSG_ N-GQDs_Ag NPs-500 and (j) Low magnifications and (k) high magnifications and (l) EDX of LSG_ N-GQDs_Ag NPs-1000. The scanning electron microscope uses a focused beam of high-energy electrons up to 30 kV.

Figure 3: XRD image of (a) LSG, (b) N-GQDs, (c) Ag NPs, (d) LSG_ N-GQDs_Ag NPs-50; (e) LSG_ N-GQDs_Ag NPs-100; (f) LSG_ N-GQDs_Ag NPs-500 and (g) LSG_ N-GQDs_Ag NPs-1000. Showing the semi-crystalline peaks of fabricated nanostructures. FT-IR spectra image of fabricated (h) LSG, (i) N-GQDs, (j) Ag NPs, (k) LSG_ N-GQDs_Ag NPs-50; (l) LSG_ N-GQDs_Ag NPs-100; (m) LSG_ N-GQDs_Ag NPs-500 and (n) LSG_ N-GQDs_Ag NPs-1000. Absorption regions are shown from 500 to 3700 cm−1.

Figure 4: (a) Survey scan of XPS core level spectra taken on LSG_N-GQDs_Ag NPs-1000. The binding energy of (b) carbon, C1s; (c) nitrogen, N1s; and (d) silver, Ag 3d.

Figure 5: (a) Nyquist plot on surface modifications on SPCE electrode. (b) Analysis of the limit of detection. Different concentrations of the target (30 ng/mL to 30 fg/mL) through impedance spectroscopy analysis were shown. (c) Impedance spectra measurement. It is to identify the selectivity of the biosensor. Human serum in the presence of other biomolecules was tested.

Figure 6: (a) Linear regression curve. Different concentrations of the target with a linear equation of ΔRct; (b) A bar diagram on the selectivity of the biosensor and its Rct value; (c) Reproducibility of the biosensor using 5 different SPE electrodes (d) Stability of the biosensor. Tested for 6 weeks against Rct is shown.