

Bioinspired porous and electroactive reduced graphene oxide hydrogel-based biosensing platform for efficient detection of Tumor Necrosis Factor- α

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Abstract

Oral cancer is one of the leading cancer types, which is frequently diagnosed at an advanced stage, giving patients a poor prognosis and fewer therapeutic choices. To address this gap, exploiting biosensors utilizing anti-biofouling hydrogels for early-stage oral cancer detection in non-invasive body fluids is gaining utter importance. Herein, we have demonstrated the fabrication of an innovative electrochemical immunosensor for the rapid, label-free, non-invasive, and affordable detection of Tumor Necrosis Factor- α (TNF- α), a biomarker associated with oral cancer progression in artificial saliva samples. The gold screen-printed electrodes (gSPE) are modified with green synthesized porous, and electroactive reduced graphene oxide (rGO) hydrogel utilizing L-cystine (L-cys) as both *in-situ* reducing and surface functionalization agent, followed by covalent immobilization of anti-TNF- α and blocking of residual sites with bovine serum albumin (BSA) to fabricate the BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensing platform. The fabricated platform demonstrates excellent performance, with a low limit of detection of 1.20 pg/mL, a broad linear range from 1 to 200 pg/mL, and a high sensitivity of 2.10 $\mu\text{A pg}^{-1} \text{ mL cm}^{-2}$ carried out with differential pulse voltammetry (DPV) technique. Moreover, it exhibits specificity towards TNF- α , even in the presence of potential interferents and other cancer biomarkers. Besides, the biosensor showed good reproducibility as well as repeatability with a relative standard deviation (%RSD) of 5.11% and 1.85%, respectively. Thus, integrating L-cys_rGO hydrogel in the immunosensor design offers enhanced performance, paving the way for its application in early-stage oral cancer diagnosis.

Keywords: Electrochemical immunosensor, L-cys, porous hydrogels, TNF- α , screen printed electrodes, artificial saliva

1. Introduction

Cancer poses a significant global health challenge, being a primary contributor to mortality rates and a significant obstacle to the global endeavor of prolonging human lifespan¹. Among them, oral cancer ranks as the sixth most prevalent form of cancer², with approximately 378,000 cases recorded worldwide in 2020³. It has a strong foothold in countries undergoing significant economic changes⁴. Oral cancer usually develops in the buccal cavity and oropharynx, with oral squamous cell carcinoma (OSCC) constituting over 90% of cases of cancers within the head and neck region⁵. Factors that contribute to the development of oral cancer encompass various etiological agents, with smoking and alcohol consumption being the foremost risk factors⁶. Despite the advancements in diagnostic technologies and easily accessible buccal cavities, a substantial number of oral cancer cases are diagnosed in advanced stages, which leads to a reduction in the chances of effective treatment. Furthermore, the 5-year survival rates for oral cancer cases that are detected in later stages have been observed at below 30%, while those detected at an early stage are above 80%, underscoring the critical importance and urgent need for early detection and intervention of oral cancer⁷.

Though current diagnostic approaches for oral cancer, such as tumor imaging, biopsy analysis, proteomics analysis, and enzyme-linked immunosorbent assay (ELISA), are there, but due to invasive, time-consuming, painful procedures, or highly trained personnel requirements, their usage is restricted⁸⁻¹⁰. Moreover, these are often applicable only in later stages of cancer progression, where substantial tumor growth has already occurred. To address this diagnostic gap, detecting biomarker levels indicative of cancer development using biosensors could be a promising alternative¹¹. Among various oral cancer biomarkers like Interleukins 6 (IL-6)¹², IL-8¹³, MMP-9¹⁴, CYFRA-21-1¹⁵, and Tumor Necrosis Factor-Alpha (TNF- α)¹⁶, which play crucial roles in oral cancer progression, TNF- α has gained significant attention in recent times. Furthermore, it is also a considerable biomarker in stress diagnosis whose increase levels makes it a valuable indicator for stress related disease. TNF- α is a 17 kDa, trimeric, pleiotropic, pro-inflammatory cytokine with significant involvement in various

inflammatory, metabolic, infectious, and neoplastic diseases. TNF- α serves as a critical mediator and has a central role in initiating immune response¹⁷. It is primarily secreted by activated macrophages but can also be synthesized by other cells, including muscle, fibroblast, and endothelial cells¹⁸. Numerous research studies have highlighted an association between elevated TNF- α levels in body fluids and the advancement of oral cancer^{19, 20}. TNF- α is implicated in promoting the survival and proliferation of cancer cells, facilitating angiogenesis and metastasis²¹. The salivary concentration of TNF- α typically measures around 30 pg/mL in individuals with oral cancer, compared to approximately 3 pg/mL in healthy subjects²².

Nowadays, biosensors have demonstrated an effective route for fast and effective biomarker quantification, facilitating early detection as well as assessment of cancer treatment efficacy^{23, 24}. Among these biosensors, electrochemical biosensors, offer an efficient way of biomarker level detection with improved speed, sensitivity, specificity, and economics of the diagnosis system^{25,26}. One key advantage of the electrochemical biosensor platform is its adaptability, allowing modifications with different transducer materials to improve the characteristics of the sensor^{27,28}. Over the last decade, extensive research has been directed towards developing novel and innovative transducer materials for electrochemical biosensors, which can uphold a good catalytic performance along with enhanced stability^{25,27,29,30}.

Nanostructured graphene-based hydrogels are garnering considerable attention as transducer materials for electrochemical biosensors³¹. However, the utility of graphene and its derivatives in hydrogels is hindered by its limited water dispersibility³². Nevertheless, the reduction of graphene oxide (GO) has been shown to improve this dispersion within a polymeric matrix and enhance performance by providing exciting electrochemical properties and a larger surface area with good functionalization capability. Reduced graphene oxide (rGO) that possesses a hexagonal carbon structure with inherent crystallinity along with various functional groups and defects in the structure have proved to influence the electrical properties, including mass transfer of electrons and electrochemical reactivity, potentially

enhancing the biosensor's sensitivity and assay time^{33,34}. Though, for synthesizing the rGO hydrogel, chemical functionalization of rGO could be done by utilizing widely used compounds like (3-aminopropyl) triethoxysilane (APTES)³⁵, oleic acid, polyethylene glycol, and 3-mercaptopropyltriethoxysilane³⁶. However, such chemicals require an additional step for functionalization and may affect human health adversely^{37,38}. To address these issues, biomolecules such as amino acids that are non-toxic in nature, are emerging for functionalization of nanomaterials and immobilization of preferred biomolecules. L-cysteine (L-cys) is a natural and easily available proteinogenic amino acid having amino (-NH₂), carboxyl (-COOH), and sulfur (-S) groups, which render it as a suitable linker for the functionalization of nanomaterials^{39,40}. Functionalizing rGO hydrogel with L-cys can provide not only increased loading of the desired antibody molecules but also covalent attachment of hydrogel to the gold surface, owing to the functional groups like amine (-NH₂) and thiol (-SH), which further increases the stability of the biosensing platform⁴¹.

In this study, we have demonstrated, the green synthesis of L-cys functionalized rGO hydrogel using one pot method and further used for the fabrication of an electrochemical immunosensor to quantify TNF- α biomarker in artificial saliva sample. The porous L-cys functionalized rGO hydrogel provided excellent conductivity along with an enhanced surface-to-volume ratio, improving the overall electrochemical performance. Besides, the bifunctional L-cys molecules not only reduce GO to rGO but simultaneously act as the bridging as well as functionalization agent to facilitate the assembly of rGO sheets into porous nanostructures. A series of techniques, including field electron scanning electron microscopy (FESEM), high-resolution transmission electron microscopy (HRTEM), Fourier transform infrared spectroscopy (FTIR), Brunauer-Emmett Teller (BET) and X-ray diffraction (XRD), have been utilized to study the morphology, structure, and functionalization of the rGO hydrogels. Leveraging the porosity, anti-biofouling nature, high electrical conductivity, and expansive surface area of L-Cys_rGO hydrogel, the fabricated biosensor demonstrated good sensitivity, high selectivity, broad linearity, and a very low detection limit for TNF- α detection.

2. Experimental Section

The section describing the “Chemicals and reagents” as well as “Instrumentation and characterizations” are fully explained in Supporting Information (SI) under subsections S1 and S2, respectively.

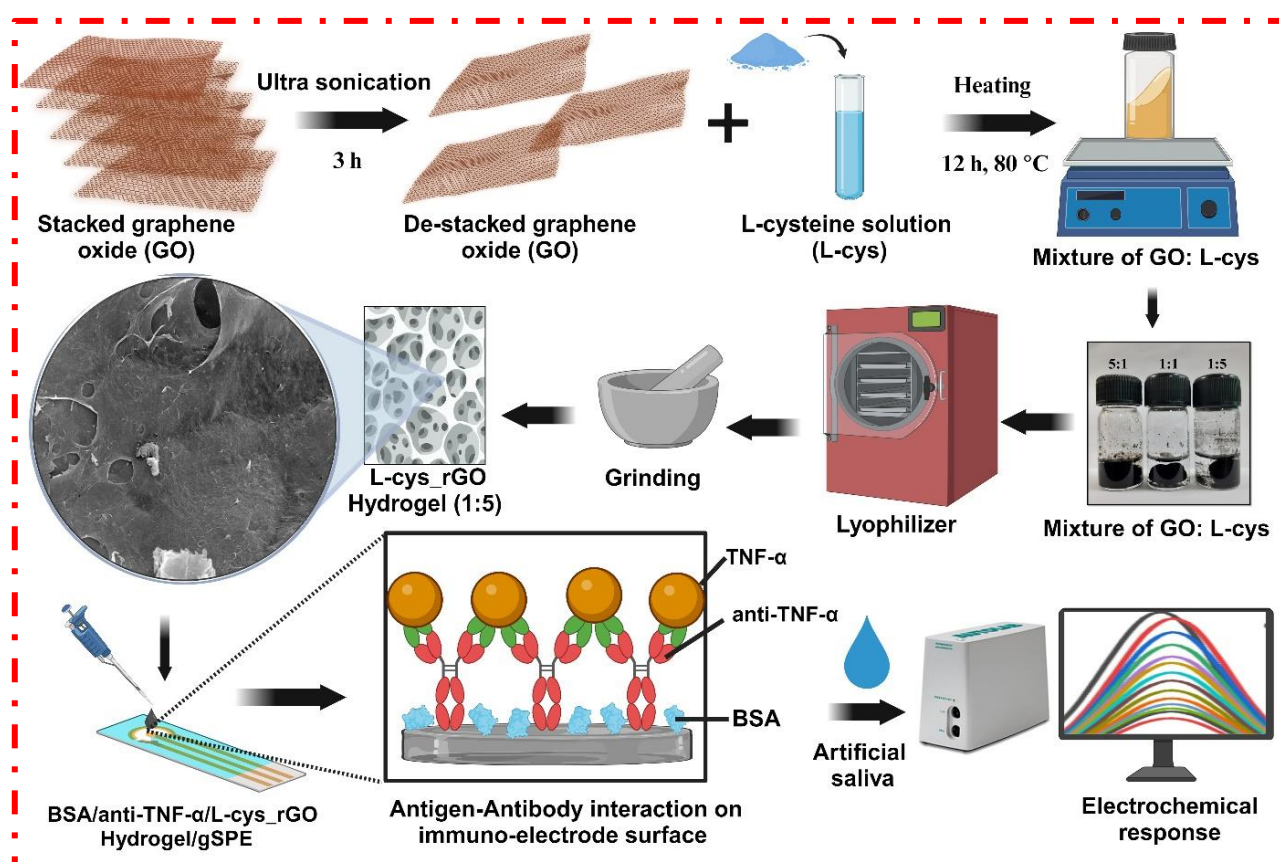
2.1 Synthesis of L-cysteine functionalized rGO (L-cys_rGO) hydrogel

L-cys functionalized rGO hydrogel was synthesized by a simple coprecipitation method utilizing L-cys as both *in-situ* reducing and surface functionalization agent⁴². Different ratios of GO: L-cys (5:1, 1:1, and 1:5) were used to optimize the process of reduction and synthesis of hydrogel. For preparing L-cys_rGO hydrogel, a 3 mg/mL dispersion of GO was firstly prepared by adding 30 mg GO to 10 mL deionized water, followed by bath sonication for around 3 h and were transferred to a 15 mL vial. For the reduction of GO along with the simultaneous formation of hydrogel, L-cys in different amounts (6, 30, and 150 mg in ratios 5:1, 1:1, and 1:5, respectively) was added dropwise to the GO dispersion. The resulting solutions were then kept overnight at 80 °C for hydrogel formation, followed by washing with deionized water and ethanol. Following the completion of hydrogel formation, the hydrogels were washed thrice using centrifugation. Initially, 5 mL of deionized water was added to the hydrogels. Then, they were centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was discarded after centrifugation. The previous step was repeated two more times, once with deionized water and then finally with ethanol. The resultant hydrogels were then lyophilized at -80 °C for 8 h and crushed to obtain the L-cys_rGO powder.

2.2 Fabrication of BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor

The immunosensing platform was fabricated using the freeze-dried L-cys_rGO hydrogel powder with a 1:5 (GO: L-cys) ratio. A 5 mg/mL suspension of L-cys_rGO hydrogel powder was made in deionized water, followed by probe sonication for 15 min. After sonication, 5 μ L of this suspension was drop casted onto the working area of the gSPE and left to dry overnight at 37 °C. The TNF- α antibody was immobilized onto the working electrode using EDC-NHS chemistry for covalent linking of the

antibodies to the hydrogel-modified gSPE. For this, a 50 $\mu\text{g/mL}$ stock solution of anti-TNF- α antibody was made in PBS (pH 7.0). Later, this anti-TNF- α antibody solution was mixed with (NHS) (0.1 M) and (EDC) (0.4 M) in a ratio of 2:1:1, respectively, and was kept under humid conditions for 45 min to activate the solution. Then, 5 μL of the activated antibody solution was dropped onto the working area of L-cys_rGO hydrogel/gSPE. It was then kept at 4 $^{\circ}\text{C}$ for 6 h in a humid chamber. During this incubation, a covalent amide bond formed between the amine group in the L-cys_rGO hydrogel and the carboxyl group of anti-TNF- α antibodies, resulting in the covalent immobilization of antibodies. The immunoelectrodes were then washed with PBS. Further, to avoid non-specific attachment of the antigen to the hydrogel surface, the free sites devoid of anti-TNF- α antibody were blocked using 2% BSA solution, fabricating BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensing platform (**Scheme 1**). After blocking, the working electrode surface was again washed with PBS to remove the excess BSA.



Scheme 1: Synthesis of L-cys_rGO hydrogel and fabrication of BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor.

3. Results and Discussions

The synthesized L-cys_rGO hydrogels are depicted in **Fig. 1**. The suspensions of GO, upon reduction by L-cys, became black and viscous. This black hydrogel mass formation could be due to the bonding between L-cys molecules and the GO surface with bonds such as C–S, S=O, and amido bonds, which led to the bridging of the nanosheets⁴². Initially, in reaction with a 5:1 ratio of GO: L-cys, a non-fluidic hydrogel with weak crosslinking can be observed. With an increase in the L-cys concentration, typical rGO hydrogel formation and precipitation from the solution could be observed, suggesting increased crosslinking of rGO by L-cys. For further analysis and immunosensor fabrication, L-cys_rGO hydrogel with a 1:5 (GO: L-cys) ratio was used, as it showed a well-formed hydrogel structure, which is confirmed using different analytical techniques including XRD, FTIR, FESEM, BET and HRTEM.

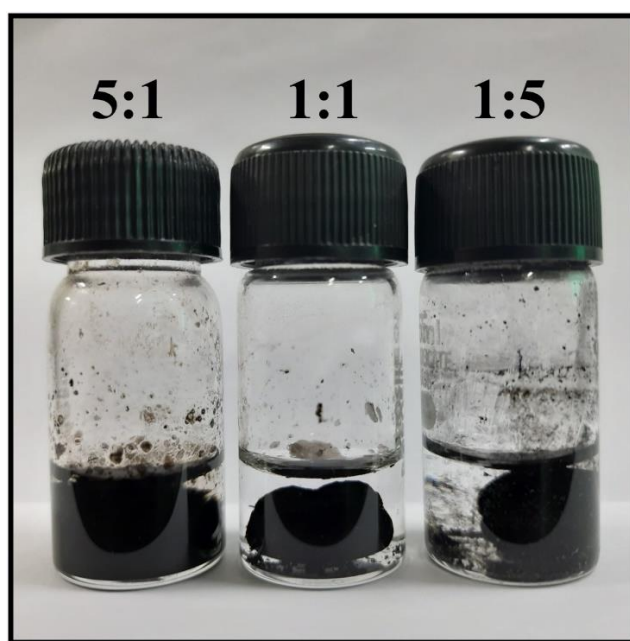


Fig. 1: Synthesis of L-cys_rGO hydrogel using three different ratios of GO: L-cys (5:1, 1:1, and 1:5).

3.1 Structural and morphological characterizations

The XRD diffraction plots (**Fig. 2**) illustrate the phase and the crystallographic structure of the materials within the 2θ range from 10° to 80° at a scan rate of 5° per min including (i) GO, (ii) L-cys,

(iii) L-cys_rGO hydrogel (5:1), (iv) L-cys_rGO hydrogel (1:1), and (v) L-cys_rGO hydrogel (1:5). In **Fig. 2(i)**, the XRD plot of GO show a characteristic peak at $2\theta = 10.8^\circ$ corresponding to (001) plane due to the graphitic nature⁴³, while in case of **Fig. 2(ii)**, the XRD plot of L-cys is shown depicting peaks at $2\theta = 12.9^\circ, 18.1^\circ, 19.5^\circ, 20.9^\circ, 21.7^\circ, 24.4^\circ, 28.3^\circ, 31.2^\circ, 32.9^\circ$ and 33.8° that corresponds to (110), (120), (101), (111), (210), (121), (211), (221), (002) and (310), respectively, that were consistent with the monoclinic system⁴⁴. Moreover, XRD patterns of the freeze-dried hydrogels [**Fig. 2(iii to v)**] show the disappearance of the characteristic peak at $2\theta = 10.8^\circ$ that revealed the successful reduction of GO into rGO by L-cys. Also, the appearance of new peaks in the curve (iii) of L-cys_rGO hydrogel (5:1) showed a broad peak at $2\theta = 22.1$ and 43 that belongs to the (002) and (001) plane, respectively⁴⁵. This is because the content of GO in this hydrogel is more as compared to L-cys, showing no extra peaks of L-cys; however, in the case of **Fig. 2(iv and v)**, the appearance of additional peaks of L-cys occurred due to equal and more content of L-cys, respectively as compared to GO. Furthermore, in L-cys_rGO hydrogel (1:5) [**Fig. 2(v)**], the incorporation of L-cys has led to the diminishing of the broad peak of rGO, forming a cross-link to form a fully 3D porous network structure.

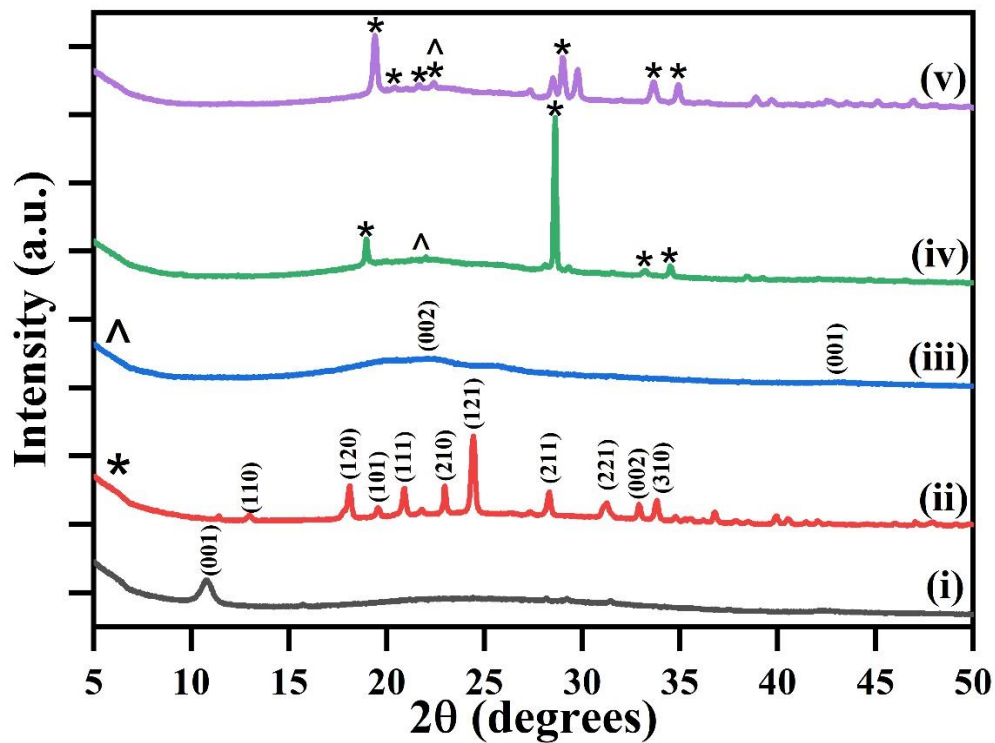


Fig. 2: XRD spectrum of (i) GO, (ii) L-cys, and L-cys_rGO hydrogels with varying GO: L-cys ratios (iii) 5:1, (iv) 1:1, and (v) 1:5.

The surface modifications of the L-cys_rGO hydrogels, GO, L-cys, and the fabricated bioelectrodes were studied using FTIR analysis. **Fig. 3(i)** demonstrates the characteristic peaks of GO at 1036 cm^{-1} , 1220 cm^{-1} , 1399 cm^{-1} , 1617 cm^{-1} , 1720 cm^{-1} , and 3160 cm^{-1} that are consistent with C-O peak, C-O-C bending, C-OH, C=C, C=O, and O-H stretching respectively⁴⁶. Also, a broad peak from $3160\text{--}3595\text{ cm}^{-1}$ corresponds to the stretching of the O-H bond in the C-OH group and water in the material⁴⁷. The 2996 cm^{-1} and 2584 cm^{-1} peaks correspond to the -NH_2 and -SH groups of cysteine attached to the rGO hydrogel⁴². Further, the peak at 1724 cm^{-1} belongs to the carbonyl group and is characteristic of GO. As the L-cys concentration used in the production of hydrogel was increased, the peak for the carbonyl group weakened, suggesting that a reaction happened between L-cys and GO. Next, **Fig. 3(ii)** presents the FTIR spectrum of L-Cys, showcasing the mercapto (S-H) group's peaks at 2549 cm^{-1} and 866 cm^{-1} , which are characteristic of L-cys. The other peaks at $1295\text{--}1390\text{ cm}^{-1}$, 2965 cm^{-1} , and 1578 cm^{-1} related to the symmetric C-O stretching of the carboxyl group, amino groups, and the deformation of the NH_2 group of L-cys, respectively. While in the case of L-Cys-rGO hydrogel [**Fig. 3(iii)**], the absence of peaks at 1720 cm^{-1} and 2549 cm^{-1} confirms the successful reduction of GO to rGO and formation of S-Au bond between the mercapto of L-Cys_rGO hydrogel and gold SPE, suggesting that thiol-terminated L-Cys_rGO molecules are likely attached to the gold surface of the electrode. Along with this, a reduction of peak intensity occurred with a slight shift to low wavenumber. Post antibody immobilization on the L-cys_rGO hydrogel/gSPE surface [**Fig. 3(iv)**], minor peak shifts occurred, with some additional peaks emerging at 1575 cm^{-1} and 1716 cm^{-1} which were likely due to the formation of amide I and II bonds between the antibodies and L-Cys_rGO hydrogel, indicating successful antibody attachment on the L-cys_rGO/gSPE surface. Subsequent BSA treatment [**Fig. 3(v)**] maintained the same peaks with reduced intensity and peak broadening at 1716 cm^{-1} and 1575 cm^{-1} .

confirming the blocking of the nonspecific vacant sites on the surface of immunoelectrodes due to BSA⁴⁸.

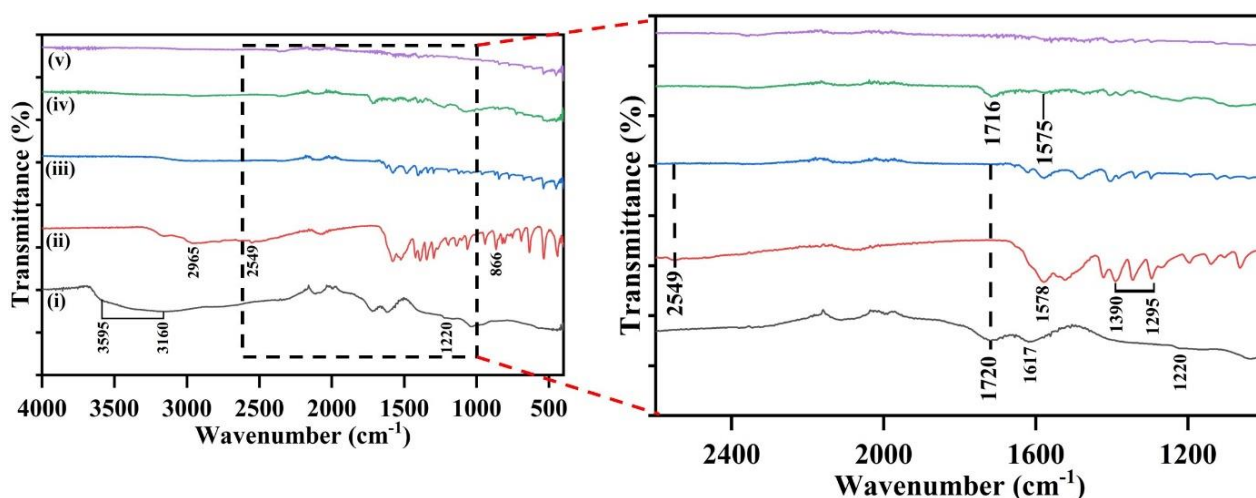


Fig. 3: FTIR spectrum of (i) GO, (ii) L-cys, (iii) L-cys_rGO hydrogel (1:5), (iv) anti-TNF- α /L-cys_rGO hydrogel/gSPE, and (v) BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE.

The morphological characterizations of L-cys_rGO hydrogel were studied by FESEM-EDX, revealing that L-Cys_rGO hydrogels exhibit porous structures. **Fig. 4(a-c)** shows the FESEM images of the surface of the L-Cys_rGO hydrogels having different GO: L-cys ratios. In hydrogel with a low concentration of L-cys (5:1 and 1:1) [**Fig. 4(a) and (b)**], small pores could be observed on the surface of the rGO nanosheet. As the GO to L-cys ratio decreased from 5:1 to 1: 5, an increase in the pore size of the hydrogel was observed. This change in pore size across the hydrogels can be attributed to the phase separation occurring between water molecules and the hydrogel network. While in the case of [**Fig. 4(c)**], increasing L-cys content leads to more interconnected GO nanosheets in the polymeric network. A higher L-cys content indicates a denser network. When the concentration of L-cys significantly surpasses the quantity of GO nanosheets, the excess L-cys might have been adsorbed onto the nanosheets, enhancing the network's hydrophilicity. Consequently, the hydrogels exhibited swelling [**Fig. 4(c)**] and a further increase in pore size. Moreover, element composition was confirmed utilizing EDX [**Fig. 4(d)**] showing the presence of elements like carbon (C, 70.72%) and oxygen (O, 29.28%). Furthermore, surface changes could be observed in case anti-TNF- α /L-cys_rGO

hydrogel/gSPE [Fig. 4(e)], and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE [Fig. 4(f)], thus confirming the successful fabrication of electrodes.

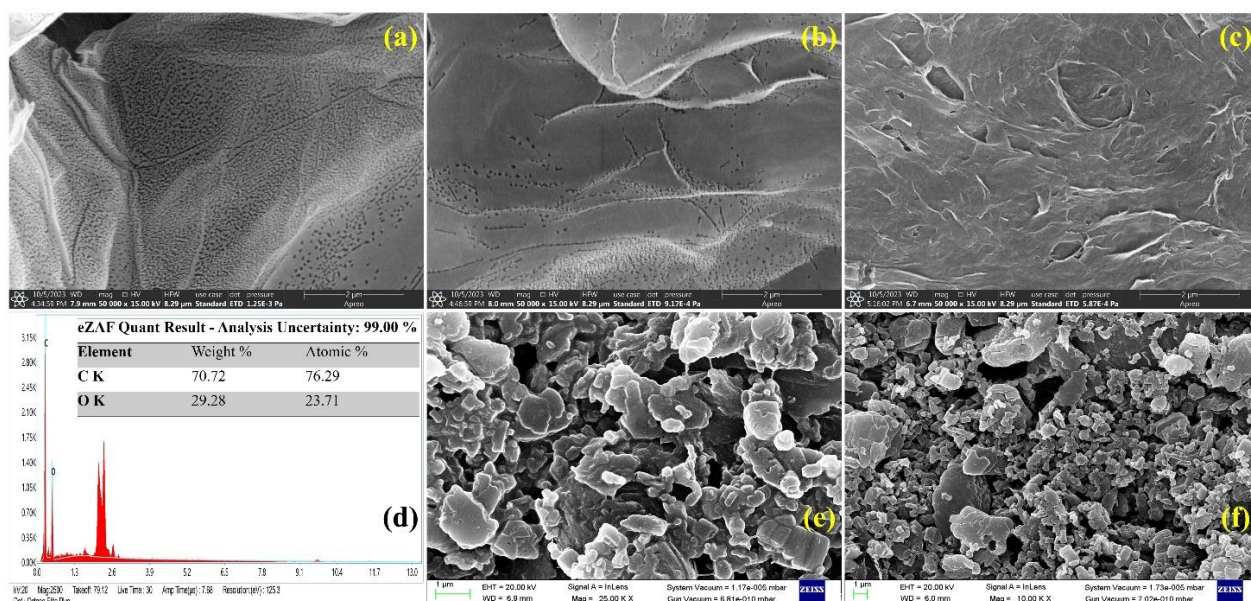


Fig. 4: FESEM of freeze-dried L-cys_rGO hydrogels of various ratio of GO: L-cys (a) 5:1, (b) 1:1, (c) 1:5, (d) EDX of L-cys_rGO hydrogel (1:5), (e) anti-TNF- α /L-cys_rGO hydrogel/gSPE, and (f) BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE.

Further, transmission electron microscopy (TEM) was used to characterize the crystalline nature and surface structures of L-cys_rGO hydrogel with GO: L-cys (1:5). The TEM images of hydrogel [Fig. 5(a-c)] shows the thin layer of exfoliated rGO along with the folding of nanosheets. The wrinkled and porous structure of L-cys_rGO hydrogel could be observed, confirming the crosslinking between the nanosheet through L-cys. Further, the SAED pattern [Fig. 5(d)] confirms the crystalline structure of rGO ascribed to the (002) reflection planes⁴⁹. The FESEM and TEM characterizations signify the successful reduction and formation of L-cys_rGO hydrogel using the one-step coprecipitation method.

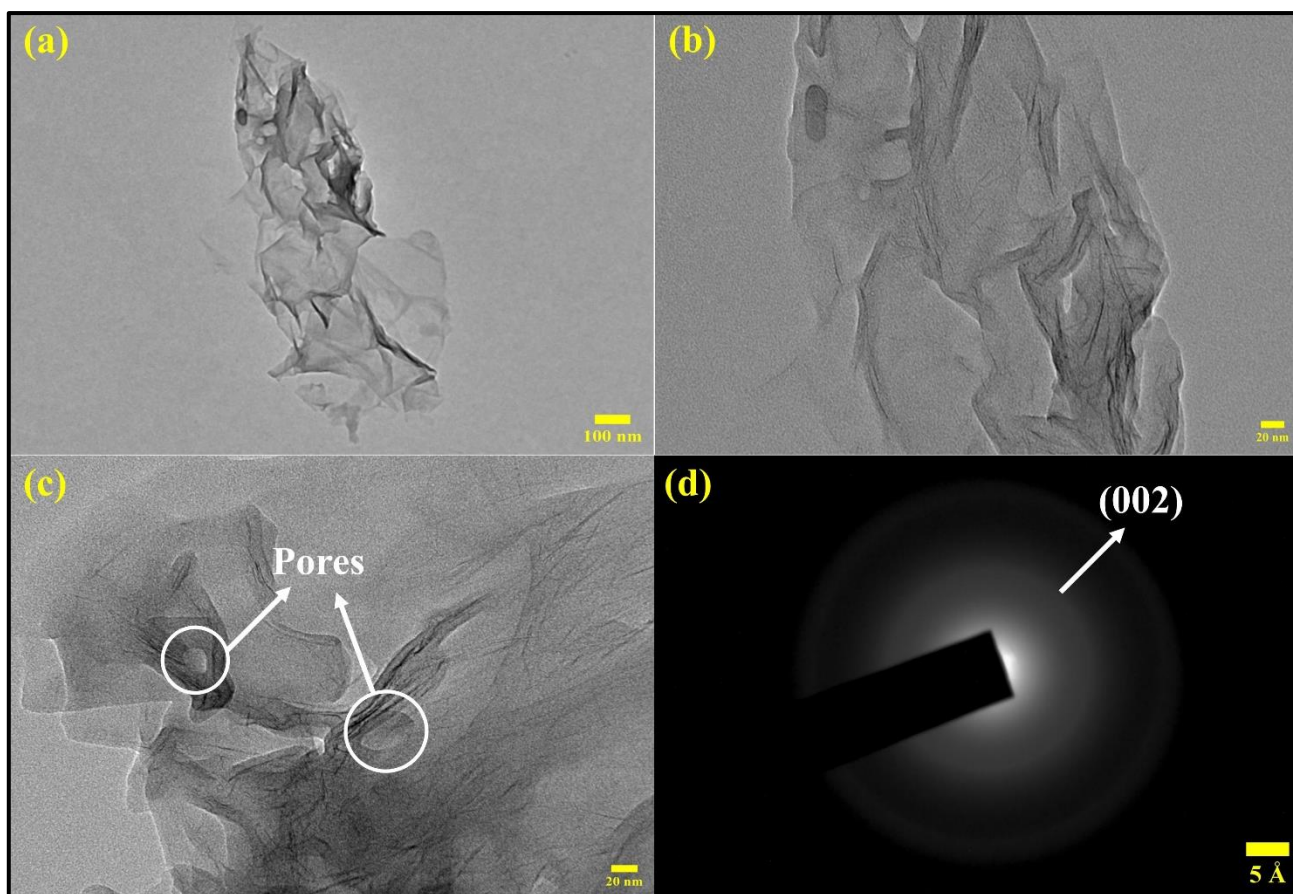


Fig. 5:(a-c) TEM of freeze-dried L-cys_rGO hydrogels with GO: L-cys (1:5), and (d) SAED pattern of L-cys_rGO hydrogel (1:5).

The nitrogen (N_2) adsorption-desorption isotherm recorded at 77.35 K was used to analyze the pore configuration of the L-cys_rGO hydrogel, including the specific BET surface area, average pore radius, and total pore volume. **Fig.6 (a) and (b)** illustrate the pore size distribution as well as the N_2 adsorption-desorption isotherm, respectively. The average pore diameter and total BET surface area were determined using the Barrett–Joyner–Halenda (BJH) approach, yielding values of $36.87 \text{ m}_2 \text{ g}^{-1}$ and 3.92 nm , respectively. Additionally, the pore volume was calculated as 0.052 cc g^{-1} . The isotherm illustrated in **Fig.6 (b)** displays an H3-type hysteresis loop which is characteristic of type-4 behavior. The characteristics indicate that the nanomaterial possesses a mesoporous surface, aligning with morphology studies such as FESEM and TEM, which reveal numerous pores within the material ^{50,51}.

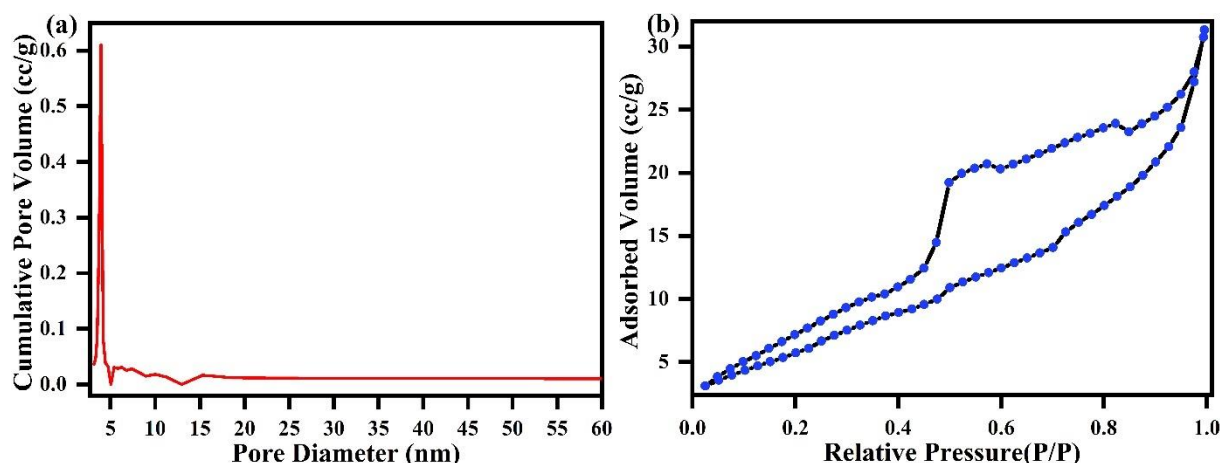


Fig. 6: (a) Pore size distribution and (B) N₂ adsorption-desorption isotherm of L-cys_rGO hydrogel with GO: L-cys (1:5).

3.2 Electrochemical studies

3.2.1 Electrode and electrokinetic studies

All the electrochemical studies of fabricated electrodes were done using phosphate-buffered saline (PBS) (0.2 M) (pH 7.0) solution infused with 5 mM of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the redox coupler. PBS of pH 7.0 was used for the whole studies because biomolecules like antibodies have the highest activity in the pH range of 7- 7.4. If pH varies too much, then antibodies can get denatured, which could lead to a loss in the specificity of the antibody towards the antigen. Differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were employed to study the electrochemical behavior of the fabricated immunoelectrodes. **Fig. 7a** shows the CV curves of (i) unmodified bare gSPE, (ii) L-cys_rGO hydrogel/gSPE, (iii) anti-TNF- α /L-cys_rGO hydrogel/gSPE, and (iv) BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE across a potential range of -0.8 to +0.8 V. The anodic peak current observed for the bare gSPE was 78.61 μA [**Fig. 7a(i)**]. However, this peak current increased to 91.34 μA for the L-cys_rGO hydrogel/gSPE [**Fig. 7a(ii)**], indicating enhanced electron transfer at the L-cys_rGO hydrogel/gSPE-electrolyte interface²⁵. It shows the good electrocatalytic abilities of rGO hydrogel. The anodic peak current decreased further with the immobilization of antibodies and BSA onto the electrode, which might be because of the non-conductive nature of the respective biomolecules³⁰. The

peak anodic current in the case of anti-TNF- α /L-cys_rGO hydrogel/gSPE [Fig. 7a(iii)] and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE [Fig. 7a(iv)] were 87.68 μ A and 77.88 μ A, respectively. A similar trend was monitored in DPV measurements [Fig. 7(b)] for (i) unmodified bare gSPE, (ii) L-cys_rGO hydrogel/gSPE, (iii) anti-TNF- α /L-cys_rGO hydrogel/gSPE, and (iv) BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE wherein the obtained peak current values were 92.13 μ A, 110.06 μ A, 83.63 μ A, and 65.10 μ A, respectively, in the potential range of -0.4 to $+0.8$ V. Table S1 of Supporting Information (SI) depicts the currents values of various electrodes obtained from CV and DPV.

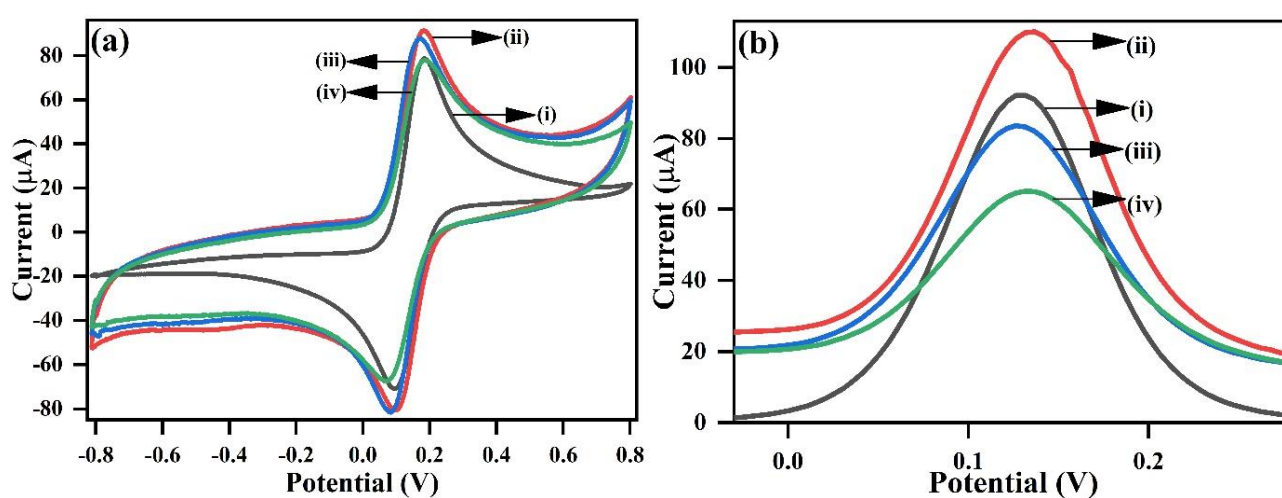


Fig. 7: (a) CV and (b) DPV responses of (i) unmodified bare gSPE, (ii) L-cys_rGO hydrogel/gSPE, (iii) anti-TNF- α / L-cys_rGO hydrogel/gSPE, and (iv) BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE.

Further, electro-kinetic analysis was performed for L-cys_rGO hydrogel/gSPE and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunoplatfroms, with different scan rates ranging across 10 to 100 mV/s to examine the various electrochemical parameters as depicted in Fig. S1 (a, and d) of SI, respectively. The resulting peak current values were displayed versus the square root of the scan rate. The anodic and cathodic peak current values were observed to exhibit a linearly increasing and decreasing behavior, respectively, with the square root of the scan rate [Fig. S1 (b and e) of SI] indicating that the electrochemical reaction adheres to a diffusion-controlled process⁵². As modifications were incrementally introduced, the peak separation increased [Fig. S1 (c and f) of SI], reflecting the occupation of the defective rapid kinetic vacancies, while the residual slow ones

accounted for the higher separation values, which reflect slower charge-transfer rates. The functionalization of L-cys_rGO hydrogel/gSPE led to an enhancement in the separation values of peak potential ($\Delta E_p = E_{p_{anodic}} - E_{p_{cathodic}}$) as shown in **Table 1** (L-cys_rGO hydrogel/gSPE = 0.196 V; BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE = 0.203 V), suggesting a slow electron transmission at the electrolyte and immunoelectrode interface.

The Brown-Anson equation⁵³ (**Eq.1**) was used for calculating the ionic species concentration (I^* in mol cm⁻²) at the surface.

$$I_p = \frac{n^2 F^2 I^* A v}{4RT} \dots\dots\dots (\text{Eq.1})$$

Wherein F represents the Faraday constant, T denotes Kelvin's room temperature, and R represents the universal gas constant (8.314 J mol⁻¹ K⁻¹). The surface ionic concentration (I^*) values for various immunoelectrodes were determined as follows: L-cys_rGO hydrogel/gSPE = 2.89 x 10⁻⁸ mol cm⁻² and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE = 2.27 x 10⁻⁸ mol cm⁻². The lower I^* value for BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE signifies the BSA functionalization on the immunoelectrode surface.

The standard heterogeneous electron transfer rate constant (K_s) plays a pivotal role in determining electron transfer rates at the electrolyte/electrode interface. To calculate the rate constant (K_s) for each immunoelectrode, the Laviron model (**Eq.2**) was utilized.⁵⁴

$$K_s = \frac{\Delta E n F V}{RT} \dots\dots\dots (\text{Eq.2})$$

Where symbols retain their previous definitions. The K_s values calculated for L-cys_rGO hydrogel/gSPE and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE platforms were found to be 0.161 s⁻¹ and 0.246 s⁻¹, respectively.

The Randles-Sevcik formula⁵⁵ was also applied to determine the coefficient of diffusion (D) for [Fe(CN)₆]^{3-/4-} redox coupler, as described in **Eq.3**.

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

Here, ‘v’ represents the scan rate (0.05 V/s), ‘D’ denotes the diffusion coefficient, ‘C’ represents the concentration of the redox coupler ($5 \times 10^{-3}\text{M}$), ‘n’ indicates the number of electrons transferred (i.e., 1), ‘A’ represents the surface area of the electrodes (0.007 cm^2), and I_p denotes the peak current of immunoelectrodes [L-cys_rGOhydrogel/gSPE = $95.54 \mu\text{A}$; BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE= $74.15 \mu\text{A}$]. Using this formula, the diffusion coefficient values were calculated for L-cys_rGO hydrogel/gSPE and BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE to be 2.02×10^{-11} and 1.24×10^{-11} respectively. The higher D-value for the former electrode suggests enhanced electron transfer at the electrolyte/electrode interface, indicating the superior analytical capability of L-cys_rGO hydrogel/gSPE.

Table 1: Scan rate derived parameters of fabricated electrodes.

Electrode	K_s	A	I^*	D	I_{pa}	I_{pc}	Ea
	(s^{-1})	(cm^2)	(mol cm^{-2})	(cm^2s^{-1})	(A)	(A)	(V)
L-cys_rGO hydrogel/gSPE	0.161	0.07	2.89×10^{-8}	2.02×10^{-11}	94.54×10^{-6}	-83.16×10^{-6}	0.196
BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE	0.246	0.07	2.27×10^{-8}	1.24×10^{-11}	74.15×10^{-6}	-62.86×10^{-6}	0.203

3.2.2 Electrochemical response study

The electrochemical performance of the immunosensor was evaluated through DPV assessment across a range of TNF- α concentrations from 0.01 to 200 pg/mL (0.01, 0.05, 1.0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 pg/mL) using PBS buffer containing 5 mM $[\text{Fe}(\text{CN})]^{3-/4-}$ as depicted by **Fig. 8 (a)**. With each increment in TNF- α concentration, a reduction was observed in the peak current. This

decreasing trend in the electrochemical behavior of immunosensors is ascribed to the formation of immunocomplex (antigen-antibody) on the electrode surface between anti-TNF- α and TNF- α concentrations, resulting in reduction in the electron transfer rate⁵⁶. **Fig. 8(b)** shows the linear relationship between the peak currents and TNF- α concentrations obtained by DPV. The regression coefficient (R^2) for this correlation is 0.979. The linear range extended from 1 to 200 pg/mL. From the curve, the following electrochemical parameters were calculated: the limit of quantification (LOQ) of 56.75 pg/mL, a sensitivity of 2.10 $\mu\text{A pg}^{-1} \text{ mL cm}^{-2}$, and the detection limit of 1.20 pg/mL. The detection limit and LOQ, and sensitivity of the fabricated immunosensing platform were calculated using standards **Eq. 4, 5, and 6**, respectively.

$$\text{Detection limit} = \frac{3\sigma}{\text{Sensitivity}} \dots\dots\dots (\text{Eq.4})$$

$$\text{LOQ} = \frac{10\sigma}{\text{Sensitivity}} \dots\dots\dots (\text{Eq.5})$$

where σ denotes the standard deviation of intercept for BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunoplatfrom estimated from linear plot.

$$\text{Sensitivity} = \frac{\text{slope of linear plot}}{\text{area of immunoelectrode (0.07)}} \dots\dots\dots (\text{Eq.6})$$

Table 2 summarizes the details of various attempts made toward electrochemical detection of TNF- α in serum and saliva samples. Most of these developed biosensors possess a very narrow detection range and high assay time. For instance, Vozgirdaite et al.⁵⁷ had a sensing range of 5-20 pg/mL. Aydin et al.⁵⁸, fabricated a biosensor based on polythiophene acetic acid with a 3.7 fg/mL LOD. However, the assay time was high (45 min), and the linear range was only 0.01-2 pg/mL. Compared to existing biosensors, our fabricated immunosensor represents a good combination of a broad linear range, a low LOD, a fast response time, and good sensitivity. Furthermore, Qi et. al. developed a biosensor for TNF- α detection with a linear range of 0.1-150 pg/mL, our biosensor demonstrates improved performance with a wider linear range of 1-200 pg/mL. Although LOD was slight lower in their case, however, our

approach offers several key advantages as it achieves a significantly fast response time of just 5 s, compared to 20 min in their study, making it faster and more efficient. Additionally, our synthesis method is green and simple, utilizing a precipitation technique, while the other approach involves complex and potentially toxic chemicals. Both sensors perform similarly, but our method is more sustainable, easier to fabricate, and holds greater potential for commercialization due to its simplicity⁵⁹.

3.2.3 Interferent study

Since any saliva sample contains numerous molecules, specificity becomes a significant characteristic of any immunosensor, which ensures that the immunosensor could detect a specific analyte from a mixture of analytes. So, to analyze the specificity of the immunoelectrode towards TNF- α , an interferent study was carried out using the DPV technique. 0.1 mM solutions of interfering agents like NaCl, KCl, CaCl₂, Cu(Ac)₂, FeSO₃, ZnNO₃, urea, glucose, fructose, and ascorbic acid, were prepared in PBS. Dilutions of other cancer biomarkers like CEA (100 ng/mL) and CYFRA-21-1 (30 μ M) were also prepared in PBS. The above-mentioned compounds were then utilized as analytes to assess their interference. DPV studies **[Fig. 8(c)]** showed that the addition of interferent analytes initially caused only a slight decrease in the peak current of the immunoelectrode, which did not change with further interferents, while the addition of TNF- α (20 pg/mL) caused a sharp decline in peak current, confirming the specificity of the fabricated BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor. The same has been depicted using the bar graph **[Fig. 8(d)]**.

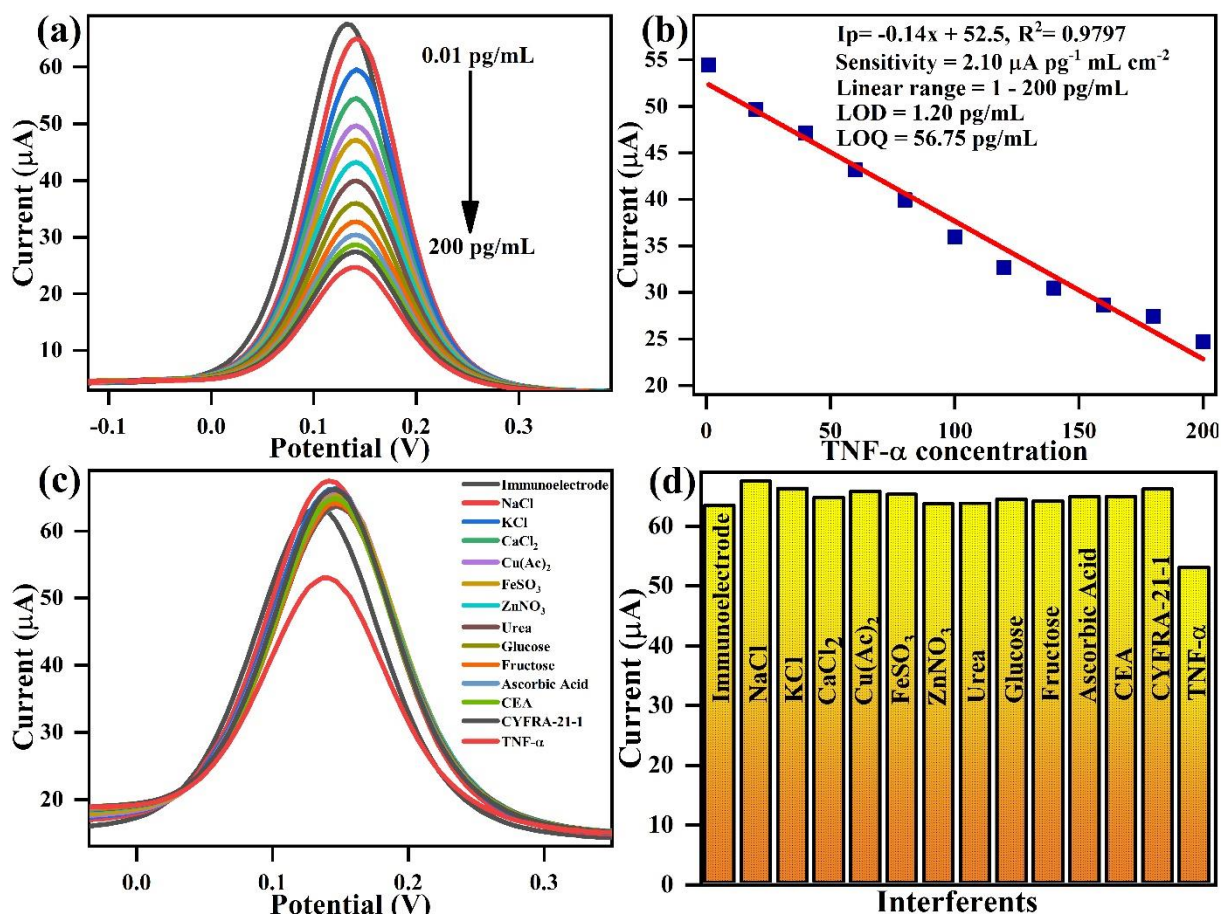


Fig. 8: The electrochemical response study of the BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor against TNF- α . (b) A linear correlation plot of the BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor demonstrates the relationship between peak currents and TNF- α concentrations. The interferent study of the BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor in PBS containing the redox species showing (c) DPV response curve and (d) Bar graph showing peak currents of BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor with different interferents.

Table 2: Summarizes the various fabricated biosensors for the quantification of TNF- α .

Electrodes	Technique	Linear Range (pg/mL)	LOD (pg/mL)	Sensitivity	Assay time	Sample	Ref.
Au WE/CMA/Ab-TNF- α /antigen-Ag-TNF- α /Ab-conjugate-HRP	Chronoamperometry	1-30	1	0.075 pg/mL	30 min	Saliva	60

TNF α -anti-TNF- Ag@Pt/MWCNTs/Chit/SPCE	Amperometry	6-60	1.6	-	>8 h	Spiked serum	61
Si ₃ N ₄ /SiO ₂ /Si[P]/Al	Capacitance	1-30	1	4.4 mV/pM	-	Artificial saliva	62
Si ₃ N ₄ ISFET/TESUD/Ab- TNF- α	Electrochemical	5-20	5	-	30 min	Artificial saliva	57
Poly(3-thiophene acetic acid) (P3)	Impedance spectroscopy	0.01-2	3.7 x 10 ³	-	45 min	Serum and saliva	58
Polymer-Coated Magnetic Microparticles onto Gold SPE	Amperometry	1 to 15	0.3	-0.116 mA/ppt	-	Artificial saliva	63
SPCE/DWCNTs/Phe-Ab- TNF/TNF α /Biotin-Ab- TNF/poly-HRP	Amperometry	1-200	0.85	-	2 h 30 min	Serum and saliva	64
Aryldiazonium and AuNPs Decorated rGO nanosheets	Amperometry	1-150	0.01	0.065 μ A/pg mL ⁻¹	20 min	Live cells	59
BSA/anti-TNF- α/L- cys_rGO hydrogel/gSPE	Amperometry	1-200	1.20	2.1	5 s	Artificial saliva	This work

3.2.4 Reproducibility, repeatability, and control studies

An important factor in the development of an electrochemical immunosensor is its reproducibility, which determines the reliability of the data obtained. The reproducibility studies [Fig. S2(a and b)] were performed on 6 electrodes separately. The electrodes were fabricated in the same conditions and had same dimensions. DPV spectra for different electrodes were noted, and the current values were plotted, with estimated relative standard deviation (%RSD) values. The current values were found to be almost similar, with slight variation. The calculated %RSD values for the electrodes are as low as 5.11%. The electrode performance showed stability for up to 3 weeks, with the maximum current decreasing marginally after the 4th week.

Similarly, a repeatability study is vital for ensuring the stability of the immunoelectrode performance during successive measurements. Fig. S2 (c and d) shows the repeatability studies with BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE in which 5 successive DPV measurements were taken for the TNF- α concentration of 1 pg/mL in PBS containing redox species. Measurements show that the peak current

values were similar for all the readings, with an %RSD of 1.85%, which confirms the reliability of the fabricated electrodes.

The control study was done using rGO/gSPE as the fabricated electrode to check and compare the biosensor performance. For this, non-functionalized rGO (5 μ L) was drop casted onto the working surface of gold SPE. The rGO/gSPE was then utilized to determine the electrochemical response by DPV against various TNF- α concentrations from 0.01 to 200 pg/mL using PBS buffer comprising 5 mM $[\text{Fe}(\text{CN})]^{3-/4-}$. We noticed a downward trend in current values till 140 pg/mL concentration of TNF- α , however on the addition of further higher concentrations, the current values started to increase. This shows an unusual trend in the biosensing performance of the platform **[Fig. S3]**. Thus, a lack of sensitivity towards TNF- α and too much variability in the readings in the control study confirms that the non-functionalized rGO/gSPE is not able to determine such a wide range of concentration of TNF- α alone with lower sensitivity. However, modification of the biosensor with BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor provides better electrochemical parameters along with good specificity towards TNF- α .

3.2.5 Spiked-sample study

To show the practical use of the fabricated BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE immunosensor platform for detection of salivary TNF- α , artificial saliva was employed as it mimics the composition of saliva. At first, addition of artificial saliva was done to observe change in current. As no current change was seen, spiked sample study was done, in which artificial saliva was spiked with known concentration of TNF- α (0.01, 0.05, 1.0, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 pg/mL). The peak current responses of these spiked samples were compared with those of standard samples with known concentrations of TNF- α in PBS, using the DPV method. **Fig. 9** shows the peak current responses of spiked artificial saliva samples and standard TNF- α concentrations. **Table S2** presents the percent recoveries (%) for spiked artificial saliva samples for different TNF- α concentrations and their average %RSD values. The %RSD values were below 5%, and %recoveries

were within the 94.15–103.55% range. The immunosensor data affirms that BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE has useful, practical applications in determining TNF- α concentrations.

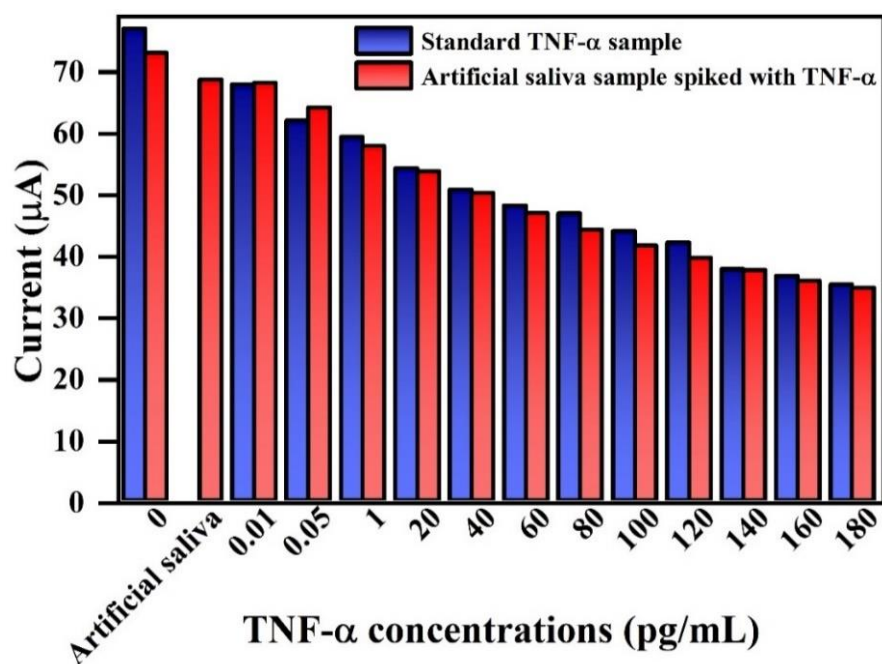


Fig. 9: Comparative bar graph showing a spiked artificial saliva sample study with standard sample utilizing BSA/anti-TNF- α /L-cys_rGO hydrogel/gSPE for TNF- α biomarker detection.

4. Conclusions

In summary, we have developed an electrochemical immunosensor platform for the easy and rapid detection of TNF- α , a salivary oral cancer biomarker. The immunosensor platform employed porous, electroactive L-cys_rGO hydrogel integrated with a screen-printed chip for TNF- α detection. The combination of L-cys_rGO hydrogel and gold electrode facilitated the rapid and efficient electron transfer formation, as well as the large surface area on the electrode, thereby enabling the covalent immobilization of anti-TNF- α antibodies at multiple sites. The increased immobilization of the antibody imparted better sensitivity to the immunosensor platform. The developed sensor demonstrated a broad detection range of 1 pg/mL to 200 pg/mL, depicting a strong linear relationship towards TNF- α concentrations and a low detection limit of 1.20 pg/mL. Our study highlights that L-cys_rGO hydrogel is a promising nanostructured transducer material due to its high surface area and porous 3D structure. The fabricated biosensor platform showed excellent experimental performance for TNF- α quantification with improved sensitivity, good reproducibility, and specificity as compared to other previous reports on TNF- α determination. Consequently, this fabricated sensor holds promise for point-of-care diagnostic (POC) applications and multiplexed biosensing by integrating with portable electronic devices, and microfluidics.

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Authors contributions

Parth Kapil: Methodology, Investigation, Data curation, Validation, Writing – original draft. **Damini Verma:** Conceptualization, Methodology, Investigation, Data curation, Validation, Writing – original draft, Formal analysis, review & editing. **Rangadhar Pradhan:** Validation, Formal analysis, writing -review & editing. **Ashish Kalkal:** Conceptualization, methodology, Formal analysis, Writing-review & editing, Supervision. **Gopinath Packirisamy:** Validation, Writing-review & editing, Resources, Supervision, Funding acquisition.

Conflict of Interest

The authors declare no conflict of interest.

Data availability

The data supporting this article have been included as part of the Supporting Information (SI).

References:

- 1 F. Bray, M. Laversanne, E. Weiderpass and I. Soerjomataram, *Cancer*, 2021, **127**, 3029–3030.
- 2 S. Warnakulasuriya, *Oral Oncol*, 2010, **46**, 407–410.
- 3 Z. W. Chan, Y. F. Phuan, P. Y. Ooi, N. Nor Azmi, D. G. S. Pateel, H. Y. Y. Yap and S. Gunjal, *BMC Oral Health*, 2023, **23**, 1–7.
- 4 D. M. Parkin, P. Pisani and J. Ferlay, *J. Cancer*, 1999, **80**, 827–841.
- 5 P. Tandon, A. Dadhich, H. Saluja, S. Bawane and S. Sachdeva, *Contemporary Oncology/Współczesna Onkologia*, 2017, **21**, 178–183.
- 6 S. Graham, H. Dayal, T. Rohrer, M. Swanson, H. Sultz, D. Shedd and S. Fischman, *JNCI: Journal of the National Cancer Institute*, 1977, **59**, 1611–1618.

- 7 C. C. R. Ragin, F. Modugno and S. M. Gollin, <http://dx.doi.org/10.1177/154405910708600202>, 2007, **86**, 104–114.
- 8 L. Acker-man, J. Del Regato, and T. Stokes. "Cancer diagnosis, treatment and prognosis." (1955): 1001.
- 9 SA Soper, K Brown, A Ellington, B Frazier, G Garcia-Manero, V Gau, SI Gutman, DF Hayes *Biosensors and Bioelectronics*, 2006•Elsevier.
- 10 R. Goldoni, M. Farronato, S. C.-B. and and undefined 2021, *ElsevierR Goldoni, M Farronato, ST Connelly, GM Tartaglia, WH YeoBiosensors and Bioelectronics*, 2021•Elsevier.
- 11 S. N. Topkaya, M. Azimzadeh and M. Ozsoz, *Electroanalysis*, 2016, **28**, 1402–1419.
- 12 G. Yang, Z. Xiao, C. Tang, Y. Deng, H. Huang and Z. He, *Biosens Bioelectron*, 2019, **141**, 111416.
- 13 N. Pachauri, G. B. V. S. Lakshmi, S. Sri, P. K. Gupta and P. R. Solanki, *Materials Science and Engineering: C*, 2020, **113**, 110911.
- 14 A. Peisker, G. F. Raschke, M. D. Fahmy, A. Guentsch, K. Roshanghias, J. Hennings and S. Schultze-Mosgau, *Med Oral Patol Oral Cir Bucal*, 2017, **22**, e270.
- 15 R. Malhotra, A. B. Urs, A. Chakravarti, S. Kumar, V. K. Gupta and B. Mahajan, *Tumor Biology*, 2016, **37**, 9263–9271.
- 16 D. Tang, D. Tao, Y. Fang, C. Deng, Q. Xu and J. Zhou, *Med Sci Monit Basic Res*, 2017, **23**, 141.
- 17 T. Horiuchi, H. Mitoma, S. I. Harashima, H. Tsukamoto and T. Shimoda, *Rheumatology*, 2010, **49**, 1215–1228.
- 18 G. Epstein Shochet, E. Brook, L. Israeli-Shani, E. Edelstein and D. Shitrit, *Respir Res*, 2017, **18**, 1–12.

- 19 C. Goertzen, H. Mahdi, C. Laliberte, T. Meirson, D. Eymael, H. Gil-Henn and M. Magalhaes, *Oncotarget*, 2018, **9**, 29047.
- 20 G. Deepthi, S. R. K. Nandan and P. G. Kulkarni, *Asian Pac J Cancer Prev*, 2019, **20**, 2087.
- 21 Y. Nakano, W. Kobayashi, S. Sugai, H. Kimura and S. Yagihashi, *Japanese Journal of Cancer Research*, 1999, **90**, 858–866.
- 22 S. Sri, D. Chauhan, G. B. V. S. Lakshmi, A. Thakar and P. R. Solanki, *Electrochim Acta*, 2022, **405**, 139736.
- 23 D. Verma, N. Dubey, A. K. Yadav, A. Saraya, R. Sharma and P. R. Solanki, *Mater Adv*, 2024, **5**, 2153–2168.
- 24 A. Kalkal, S. Kadian, S. Kumar, G. Manik, P. Sen, S. Kumar and G. Packirisamy, *Biosens Bioelectron*, 2022, **195**, 113620.
- 25 S. Kumar, Ashish, S. Kumar, S. Augustine, S. Yadav, B. K. Yadav, R. P. Chauhan, A. K. Dewan and B. D. Malhotra, *Biosens Bioelectron*, 2018, **102**, 247–255.
- 26 R. Gupta, N. Salaris, A. Kalkal, P. Mandal, S. Balabani, R. Motallebzadeh and M. K. Tiwari, *IEEE Sens Lett*, 2024, 1–4.
- 27 S. Kumar and A. Kalkal, *Nanotechnology in Cancer Management: Precise Diagnostics toward Personalized Health Care*, 2021, 43–71.
- 28 A. Kalkal, A. Tiwari, D. Sharma, M. K. Baghel, P. Kumar, R. Pradhan, and G. Packirisamy, *Int J Biol Macromol*, 2023, **253**, 127260.
- 29 R. Gupta, N. Salaris, A. Kalkal, P. Mandal, S. Balabani, R. Motallebzadeh and M. K. Tiwari, *IEEE Sens Lett*, , DOI:10.1109/LSSENS.2024.3399604.

- 30 D. Verma, S. K. Yadav, A. Kalkal, R. Pradhan and G. Packirisamy, *IEEE Sens Lett*, 2023, **7**, 1–4.
- 31 H. S. Song, O. S. Kwon, J. H. Kim, J. Conde and N. Artzi, *Biosens Bioelectron*, 2017, **89**, 187–200.
- 32 X. Huang, X. Qi, F. Boey and H. Zhang, *Chem Soc Rev*, 2012, **41**, 666–686.
- 33 S. Cui, H. Pu, E. C. Mattson, Z. Wen, J. Chang, Y. Hou, C. J. Hirschmugl and J. Chen, *Anal Chem*, 2014, **86**, 7516–7522.
- 34 A. Kalkal, R. Pradhan and G. Packirisamy, *Int J Biol Macromol*, 2023, **242**, 125157.
- 35 V. K. S. Hsiao, J. R. Waldeisen, Y. Zheng, P. F. Lloyd, T. J. Bunning and T. J. Huang, *J Mater Chem*, 2007, **17**, 4896–4901.
- 36 A. Chen and S. Chatterjee, *Chem Soc Rev*, 2013, **42**, 5425–5438.
- 37 S. K. Vashist, E. Lam, S. Hrapovic, K. B. Male and J. H. T. Luong, *Chem Rev*, 2014, **114**, 11083–11130.
- 38 S. Tiwari, P. K. Gupta, Y. Bagbi, T. Sarkar and P. R. Solanki, *Biosens Bioelectron*, 2017, **89**, 1042–1052.
- 39 W. W. A, P. Handler, E. Smith and D. Stetten, .
- 40 K. Huang, J. Li, Y. Liu, X. Cao, S. Yu, M. Y.-M. Acta and undefined 2012, *SpringerKJ Huang, J Li, YM Liu, X Cao, S Yu, M YuMicrochimica Acta, 2012•Springer*, 2012, **177**, 419–426.
- 41 G. Yao and Q. Huang, *Journal of Physical Chemistry C*, 2018, **122**, 15241–15251.
- 42 Y. Wang, Y. Xiao, G. Gao, J. Chen, R. Hou, Q. Wang, L. Liu and J. Fu, *J Mater Chem B*, 2017, **5**, 511–516.
- 43 W. Lv and H.-M. Cheng, , DOI:10.1002/adma.200803726.

- 44 Y. Su, E. P. Hessou, E. Colombo, G. Belletti, A. Moussadik, I. T. Lucas, V. Frochot, M. Daudon, S. Rouzière, D. Bazin, K. Li, P. Quaino and F. Tielens, *Amino Acids*, 2022, **54**, 1123–1133.
- 45 H. S. Maharana, P. K. Rai and A. Basu, *J Mater Sci*, 2017, **52**, 1089–1105.
- 46 R. A. Rochman, S. Wahyuningsih, A. H. Ramelan and Q. A. Hanif, *IOP Conf Ser Mater Sci Eng*, DOI:10.1088/1757-899X/509/1/012119.
- 47 E. Andrijanto, S. Shoelarta, G. Subiyanto and S. Rifki, *AIP Conf Proc*, 2016, **1725**, 20003.
- 48 S. Tiwari, P. K. Gupta, Y. Bagbi, T. Sarkar and P. R. Solanki, *Biosens Bioelectron*, 2017, **89**, 1042–1052.
- 49 D. Panda, A. Nandi, S. K. Datta, H. Saha and S. Majumdar, *RSC Adv*, 2016, **6**, 47337–47348.
- 50 M. Thommes, K. Kaneko, A. V. Neimark, J. P. Olivier, F. Rodriguez-Reinoso, J. Rouquerol and K. S. W. Sing, *Pure and Applied Chemistry*, 2015, **87**, 1051–1069.
- 51 Y. Lu, Q. Yang, S. Wang, M. Liu, Y. Chen, Z. Zhao, A. R. Akbar, C. Xiong and G. H. Hu, *Ionics (Kiel)*, 2021, **27**, 3615–3626.
- 52 D. Verma, T. K. Dhiman, M. Das Mukherjee and P. R. Solanki, *J Electrochem Soc*, 2021, **168**, 97504.
- 53 D. Verma, R. Sajwan, G. B. V. S. Lakshmi, A. Kumar, R. K. Sajwan and P. R. Solanki, *Environmental Science: Nano*, 2022•*pubs.rsc.org*, DOI:10.1039/D2EN00624C.
- 54 D. Chauhan, A. K. Yadav and P. R. Solanki, *Microchimica Acta*, 2021, **188**:1-1. DOI:10.1007/S00604-021-04751-Y.
- 55 D Verma, D Chauhan, M Das Mukherjee, KR Ranjan, AK Yadav, PR Solanki *Journal of Applied Electrochemistry*, 2021•Springer, 2021, **51**, 447–462.
- 56 A. K. Yadav, P. Gulati, R. Sharma, A. Thakkar and P. R. Solanki, *Talanta*, 2022, **243**, 123376.

- 57 D. Vozgirdaite, H. Ben Halima, F. G. Bellagambi, A. Alcacer, F. Palacio, N. Jaffrezic-Renault, N. Zine, J. Bausells, A. Elaissari and A. Errachid, *Chemosensors 2021, Vol. 9, Page 26*, 2021, **9**, 26.
- 58 E. B. Aydın, M. Aydın and M. K. Sezgintürk, *Biosens Bioelectron*, 2017, **97**, 169–176.
- 59 M. Qi, Y. Zhang, C. Cao, M. Zhang, S. Liu and G. Liu, *Anal Chem*, 2016, **88**, 9614–9621.
- 60 L. Barhoumi, A. Baraket, F. G. Bellagambi, G. S. Karanasiou, M. Ben Ali, D. I. Fotiadis, J. Bausells, N. Zine, M. Sigaud and A. Errachid, *Sens Actuators B Chem*, 2018, **266**, 477–484.
- 61 M. Mazloun-Ardakani and L. Hosseinzadeh, *RSC Adv*, 2015, **5**, 70781–70786.
- 62 M. Bahri, A. Baraket, N. Zine, M. Ben Ali, J. Bausells and A. Errachid, *Talanta*, 2020, **209**, 120501.
- 63 L. Barhoumi, F. G. Bellagambi, F. M. Vivaldi, A. Baraket, Y. Clément, N. Zine, M. Ben Ali, A. Elaissari and A. Errachid, *Sensors 2019, Vol. 19, Page 692*, 2019, **19**, 692.
- 64 E. Sánchez-Tirado, C. Salvo, A. González-Cortés, P. Yáñez-Sedeño, F. Langa and J. M. Pingarrón, *Anal Chim Acta*, 2017, **959**, 66–73.