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ORIGINAL PAPER

A graphene electrode functionalized with aminoterephthalic acid for impedimetric immunosensing of *Escherichia coli*

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Abstract

A screen-printed electrode prepared from graphene oxide (GO) has been functionalized with 2-aminoterephthalic acid, followed by the exploitation of this functional material in an electrochemical immunoassay for *Escherichia coli (E. coli*) by immobilizing the antibody on its surface. The functionalization steps followed a straightforward approach and were proven by various instrumental techniques. The detection of *E. coli* with antibody immobilized electrodes was performed using electrochemical impedance spectroscopy. The analyses were carried out using the hexacyanoferrate redox couple as the electrochemical probe. The present method has a wide analytical range (from 2.2×10^2 to 2.2×10^8 cfu.mL^{-1}), a low limit of detection (2 cfu.mL^{-1}), fast response (4 min), and good stability (up to 2 months). The analytical performance of the biosensor was comparable to the previously reported electrochemical biosensors for *E. coli*. As such, the approach of functionalization of graphene with 2-aminoterephthalic acid should be useful to allow the development of other similar sensing systems for other environmentally and clinically important analytes.

Keywords Graphene oxide · Functionalization, 2-aminoterephthalic acid, screen-printed electrode · Biosensor · Pathogen · *E. coli*

Introduction

E. coli is one of most reported disease-causing pathogens. The consumption of *E. coli* contaminated food or water can lead to several health problems, such as diarrhoea, pneumonia, urinary tract infections, etc. Therefore, it is important to develop user-friendly tools that can allow rapid as well as sensitive detection of *E. coli*. Biosensors are particularly attractive for the quantification of bacteria (including *E. coli*) on account of their simplicity, rapid results, low-cost, user-friendliness, and high sensitivities [1, 2]. Of different categories of biosensors available, the electrochemical immunosensors have the potential to be developed as portable devices and their commercial success is also well reflected by examples like glucometer, troponin analysers, etc.

Transducer surface is an important component of an electrochemical biosensor. An efficient transducer material is the key to define some critical performance parameters, e.g. sensitivity, biomolecule stability, and accuracy. In recent years, graphene and its derivatives have shown great significance in the development of electrochemical biosensors [3, 4]. Graphene is a 2-dimensional (2D) nanostructured materials with high electron mobility and surface area. These qualities of graphene make it particularly useful in electrochemical biosensors for a variety of analytes, including bacteria. For instance, an electrode modified with reduced graphene oxide (rGO)-polyethyleneimine (PEI) composite has provided the detection of *E. coli* with a low limit of detection (LOD) of 10 cfu.mL^{-1} [5].

Most of the graphene based electrochemical biosensors employ this material in its reduced form, i.e., rGO. Nonetheless, GO based biosensing platforms must also be explored despite of fact that GO is not as conducting as rGO. GO can offer more reproducible electronic properties

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and higher density of functional groups for the immobilization of biorecognition molecules. The previous examples of GO based electrochemical biosensors include the systems for the analysis of glucose, organophosphate pesticides (OPs), *E. coli*, carcinoembryonic antigens (CEA) [6, 7].

This works reports the development of a GO based electrochemical immunosensor for *E. coli*. The use of antibodies in the development of detection systems for bacteria has been advocated with advantages of high specificity and sensitivity. Unlike other biorecognition molecules such as aptamers, DNAs, and bacteriophages, the antibodies are frequently available as commercial product. GO has been functionalized with 2-aminoterephthalic acid to introduce amine functionality. This approach for a convenient and robust functionalization of GO is being reported for the first time, and as our studies have highlighted, the resulting NH_2-GO surface has facilitated attachment of antibodies. Based on the electrochemical impedance spectroscopy technique (EIS), the antibody immobilized NH_2-GO electrode has been found highly sensitive toward specific detection of *E. coli.*

Materials and methods

Chemicals and characterization tools

All the chemicals were high purity products purchased from Sigma (India) or Merck (India) (https://www.sigmaaldrich.com). Phosphate buffered saline (PBS, 10× concentrate) was also purchased from Sigma and diluted to 1× before use (pH = 7.4). Anti-*E. coli* O157:H7 antibodies were purchased from Abcam (India) (https://www.abcam.com/). The screen-printed electrodes (carbon SPEs, surface area of working electrode = 0.1256 cm^2) were procured from Zensor, Taiwan (https://www.zensor.com/tw). The bacterial strains, *E. coli and S. aureus,* were procured from Microbial Tissue Culture and Gene Bank (MTCC) of Institute of Microbial Technology (IMTECH), Chandigarh, India. *S. arlettae* was isolated in the laboratory as per the protocol developed earlier [8]. Colony-forming unit count method was used for the enumeration of culturable cells.

Spectroscopic characterizations of samples were carried out using UV-Visible (Cary 7000, Agilent, USA, https://www.agilent.com), infrared (FTIR, Spectrum II Perkin Elmer, USA, https://www.perkinelmer.com), and Raman (InVia Renishaw, UK, https://www.renishaw.com) spectroscopes. The structural and morphological investigations were carried out on X-ray diffractometer (D8 Advance, Bruker, Germany, https://www.bruker.com) and a scanning electron microscope (FE-SEM, SE 4200, Hitachi, Japan, https://www.hitachi-hightech.com). The electrochemical studies were carried out on an electrochemistry instrument (CHI 660C, USA, https://www.chinstruments.com/). The modelling of data in a suitable equivalent circuit and the estimation of charge transfer resistance (R_{ct}) values were processed with the help of a trial version of ZView software.

Synthesis of graphene oxide (GO) and its functionalization

GO was prepared by following the modified Hummer's method [9], as per details provided in the Supplementary Material. For amino-functionalization, two separate solutions, containing 250 mg of GO and 125 mg of 2-aminoterpthalic acid, both in 50 mL of water were prepared via stirring (30 min). The above two solutions were mixed and then vigorously stirred for 60 min (50 °C) to form a greenish-black precipitate. The synthesized functionalized composite was separated from the reaction mixture after centrifugation at 10000 rpm (15 min). The composite was washed several times to remove unwanted impurities. At the end, the product was dried overnight at 50 °C under vacuum and stored for further use.

Casting of amino-functionalized GO (NH_2-GO) on screen-printed electrodes and subsequent covalent attachment of anti-*E. coli* antibodies

A suitably diluted slurry of amino-functionalized GO (NH_2-GO) composite was drop casted onto the working electrode areas of screen-printed electrodes. Several of such modified SPEs were prepared and dried under vacuum (at 40 °C for about 1 h) to ensure robustness of the composite attachment on the electrode surface. The immobilization of anti-*E.coli* antibodies (Ab) on the NH_2-GO modified SPEs (NH_2-GO/SPEs) was achieved via covalent bonding. For this, the NH_2-GO/SPEs electrodes were incubated with a mixture of 0.05 M EDC [(1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide] and 0.01 M NHS (N-hydroxysuccinimide) in PBS for 2 h. Due to the porous nature of GO and its modification with terephthalic acid, large number of free -NH_2 groups were available for the immobilization of antibodies. For antibody immobilization, the NH_2-GO/SPEs, activated with EDC, were left in contact (1 h) with 20 μL of 1 $\mu g.mL^{-1}$ anti- *E.coli* solution prepared in PBS buffer (pH 7.4). The resulting Ab/NH_2-GO/SPEs were washed with Tween-20 solution to block non-specific binding sites. Finally, the prepared bioelectrodes were washed with PBS and then stored under refrigerated conditions (4 °C).

Electrochemical measurements

Electrochemical impedance spectroscopy studies were carried out in a redox electrolyte medium, i.e., 10 mM ferro/ferricyanide ($[Fe(CN)_6]^{3-/4-}$ (hexacyanoferrate)), within a frequency range of 0.1 Hz - 1 MHz keeping an amplitude of

5 mV. The above redox electrolyte was prepared by mixing 10 mM K_3 $Fe(CN)_6$ and K_4 $Fe(CN)_6.3H_2O$ in 0.1 M PBS.

Analysis of *E. coli* in standard and unknown samples

The bacterial strains were first filtered with a 0.45 mm hydrophilic filter paper followed by their re-suspension in PBS. Thus, a stock solution of bacteria was prepared. The standard colony-forming unit counts method was used for the quantification of cells in the stock solution. Further smaller concentrations of bacteria were prepared by dilution in phosphate buffered saline. For analysis by the Ab/NH_2-GO/SPEs, the bacteria solutions (10 μL) were introduced on the working area of SPEs and left to incubate for a pre-defined period of time (1–10 min). The electrodes were subsequently washed with PBS. The EIS characteristics of the electrodes were then analysed in the hexacyanoferrate electrolyte (10 mL). The experimental conditions during the collection of EIS data have been mentioned in Sections Electrochemical Measurements and Electrochemical characterization and bioassay of *E.coli*. The electrodes' outputs (Nyquist plots) were fitted in a suitable equivalent circuit (as elaborated in Section Electrochemical..... of *E.coli*) for the estimation of charge transfer resistance (R_{ct}) values. A calibration plot was prepared between the recorded R_{ct} values and the respective bacteria concentration. The concentration of bacteria in any unknown sample could be deduced by measuring the corresponding electrode response in terms of R_{ct} value and then using the standard calibration plot to find the corresponding concentration value.

As such, an average time taken for the complete analysis of a sample was about 30 min, which included the incubation of sample with the Ab/NH_2-GO/SPEs electrodes, subsequent washing steps, collection of EIS signal, and computation of acquired data to derive the concentration values.

Results and discussion

Choice of materials

Graphene oxide has been chosen as an active electrode material, aiming the development of an electrochemical biodetection electrode for *E. coli*. Due to its 2D layered structure and favourable electron transport characteristics, GO is an attractive choice in the development of electrochemical biosensors. GO possesses readily available reactive functional groups, such as hydroxyl, carboxyl, and epoxy groups. Hence, the modification of GO surface with biorecognition molecules is easier than other 2D layered options, such as reduced GO, molybdenum disulphide (MoS_2), and conducting polymers. Further, the use of antibodies has been preferred as biorecognition probe for *E. coli*. Unlike other possible options, such as aptamers, bacteriophages, and DNAs, the antibodies against *E. coli* are a relatively low-cost alternative and well established for their specificity.

Spectrophotometric characterization of materials

The material characterization at different stages is important to verify the success of involved procedures. For instance, the FTIR spectrum of GO is shown in Fig. S1. A band around 3441 cm^{-1} corresponded to stretching vibrations of hydroxyl group. The bands around 1730 and 1630 cm^{-1} were attributed to C=O stretching of carboxyl and carbonyl groups, respectively, while those around 1226 and 1044 cm^{-1} indicated C-O stretching vibrations [10]. Several other bands between 970 and 1150 cm^{-1} referred to C-O stretching of epoxy group [11, 12]. After the amine functionalization with 2-aminoterephthalic acid, the GO sample (NH_2-GO) revealed two additional bands at 1600 cm^{-1} and 1378 cm^{-1} due to N-H and C-N stretching vibrations, respectively. A decrease in the intensity of bands between 3200 and 3500 cm^{-1} was also an indicator about the functionalization of GO [13]. FTIR spectrum, collected after the attachment of the antibodies on NH_2-GO, was characterized with a band at 1640 cm^{-1} depicting the N=C vibrations of amide bond formed between the -NH_2 of GO and -COOH of the proteinaceous antibodies.

Raman spectroscopy has also been performed on different samples (Fig. S2). Both GO and NH_2-GO showed peaks at 1350 and 1581 cm^{-1} representing graphene specific typical D and G bands, respectively [14, 15] . The D peak gives information about the defects and impurities of graphitic structure, while the G peaks refers to doubly degenerate zone centre E_{2g} mode of sp^2 carbon bonding [14]. The ratio of D to G band intensity (I_d/I_g) was almost identical for both GO and NH_2-GO samples. Therefore, it can be concluded that amine functionalization of GO with 2-amino terephthalic acid did not bring any further significant defect in the structure of graphene and -NH_2 groups got attached primarily on the already present defect sites (oxidising groups) [12] .

Figure 1a presents the UV-Vis absorption profiles of GO and NH_2-GO. In case of GO, a characteristic peak at 230 nm was attributed to the π-π transitions of aromatic C=C bonds [16, 17]. A shoulder region around 300 nm was associated with the n-π* of C = O bonds [18]. The 230 nm band displayed a red shift to 260 nm after amine functionalization (NH_2-GO) as a result of partial deoxygenation [19]. A broad peak at 350 nm was associated with the presence of 2-aminoterephlathic acid and its successful π- interaction with graphene oxide [20, 21].

Structural and morphological studies

XRD patterns of GO and NH_2-GO are presented in Fig. 1b. A characteristics peak of GO at 10° was observed, suggesting large interlayer distance. This kind of interlayer spacing was associated with the formation of hydroxyl, epoxy and

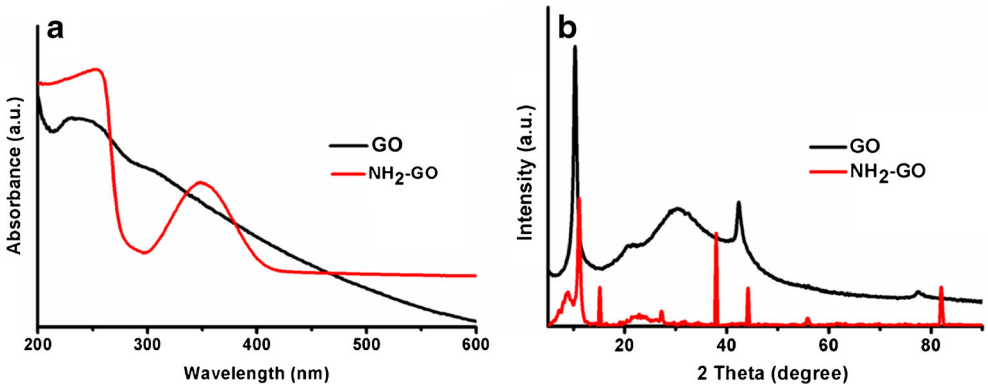

Fig. 1 Characterizations of synthesized GO and NH_2-GO samples **a** UV-Vis spectra, **b** XRD patterns

carboxyl groups on GO [22]. A broad peak at 30° can be explained by the presence of oxygen atoms that entered in the graphite gallery during the intercalation process [23]. Another peak at 42° was indicative of an incomplete oxidation of graphite [24]. In case of NH_2-GO, the intensity of 10° peak got reduced due to an introduction of amine groups between the GO layers [25]. The introduction of 2-amino terephthalic acid influenced the d-spacing and subsequently the degree of GO stacking. The reaction of amine functionalization caused limited reduction of other functional groups and the same was reflected by the sharpening of the diffraction peaks [26]. The introduction of some other peaks was associated with the presence of 2-amino terephthalic acid. The XRD patterns were fairly consistent with the previously reported data.

The morphological characterization of samples (FE-SEM images) has been shown in Fig. S3 (Supplementary Material). Both the GO and NH_2-GO samples were characterized with a chiffon sheets like morphology. A reduction in the lateral dimensions of GO sheets after functionalization can be associated to the external forces exerted by the amine groups.

Electrochemical characterizations and bioassay of *E. coli*

Electrochemical impedance spectroscopy has been used to characterize the electrodes during different steps of their preparation. Figure 2a shows the EIS investigations based Nyquist plots for bare SPE (GO/SPE), NH_2-GO/SPE, Ab/NH_2-GO/SPE and Ag/Ab/NH_2-GO/SPE (Ab = antibody, Ag = antigen). Nyquist plots were fitted in a suitable equivalent circuit (as shown in Fig. 2a), also comprising Warburg element. As the results show, the R_{ct} of the NH_2-GO/SPE (1891 Ω) was higher than that of bare SPE (754 Ω). This was expected because GO is known as a repatively less conducting material. The immobilization of antibody on the NH_2-GO/SPE surface led further increase in the R_{ct} value (1960 Ω) due to the modification of the electrode layer with biomolecules that restricted the movement of charge across the electrode-electrolyte surface [27].

After successfully characterizing the Ab/NH_2-GO/SPE electrodes, their utility was assessed for the bioassay of *E. coli*. Different Ab/NH_2-GO/SPE electrodes were exposed to varying bacterial concentrations (2.2×10^2 to 2.2×10^8 cfu.mL^{-1}) as per the details provided in experimental section. The EIS based responses of electrodes in terms of Nyquist plots were recorded and are presented in Fig. 2b. The semicircle diameter of Nyquist plots was found directly proportional to the concentration of *E. coli* tested. This behaviour could be explained on the fact that the antigen-antibody binding at the electrode surface altered the properties of dielectric layer and hindered the process of charge transfer. Moreover, the above increase of the R_{ct} can also be attributed to repulsing forces between the negatively charged biomolecules (both antibody and antigens at the electrode surface) and the negatively charged ferri/ferro redox couple.

The time required for the maximum antigen-antibody interaction at the electrode interface was also investigated. It was found that 4 min of incubation of bacterial sample with the Ab/NH_2-GO/SPE electrode was sufficient to yield a stable EIS response. The EIS responses of the different Ab/NH_2-GO/SPEs for different *E. coli* concentrations were fit in to an equivalent circuit (Fig. 2a) and assessed for the corresponding R_{ct} values. A log-log plot of R_{ct} vs bacteria concentration is presented in Fig. 2c. The results showed a linear relationship between log R_{ct} and log [*E. coli*]. Thus, it can be outlined that the Ab/NH_2-GO/SPEs are useful to detect the presence of *E. coli* in aqueous solutions over a wide concentration range. The limit of detection (LOD) was determined statistically as per the standard formula, i.e., LOD = 3 S_b/m, where, S_b was the standard deviation estimated on three blank measurements and *m* was the slope of the calibration plot. This above equation derived the LOD of the present biosensor to be 2 cfu.mL^{-1} (antilog of 0.4 and figure rounded off).

Optimization of assay parameters

The reproducibility of the Ab/NH_2-GO/SPEs electrodes has been assessed. For this, five numbers of sensor electrodes prepared under identical conditions were used to record the impedance responses toward a fixed concentration (2.2×10^4 cfu.mL^{-1}) of *E. coli* (Fig. S4). All the tested five

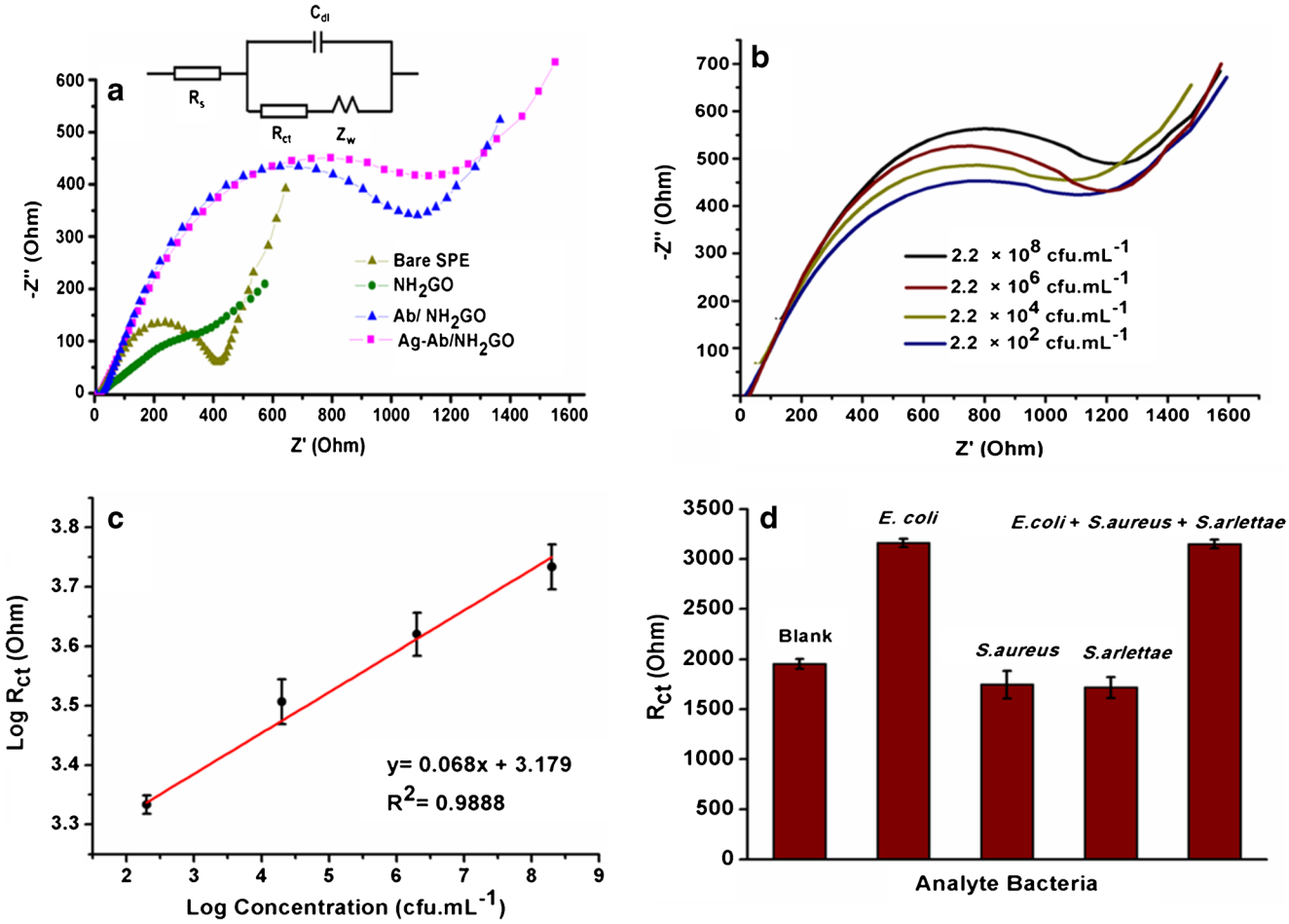

Fig. 2 **a** Nyquist plots of bare SPE, NH_2-GO, Ab/NH_2-GO/SPE and Ag-Ab/NH_2-GO/SPE; **b** Nyquist plot of the Ab/NH_2-GO/SPEs with respect to different concentrations of *E.coli* (2.2×10^2 to 2.2×10^8 cfu.mL^{-1}); **c** Calibration plot; **d**. Specificity of Ab/NH_2-GO/SPE toward *E. coli* (2.2×10^4 cfu.mL^{-1}) in co-presence of other potentially interfering bacteria, i.e., 2×10^6 cfu.mL^{-1} *S. aureus* and *S. arlettae*. General experimental conditions: Electrode-sample incubation time = 4 min, Electrolyte = 10 mM $[Fe(CN)_6]^{3-/4-}$, Frequency range = 0.1–1 MHz, AC amplitude = 5 mV

Table 1 An overview on recently reported electrochemical sensors employed for the detection of *E. coli*

S. No	Nanomaterial used in electrode	Technique	Detection range (cfu.mL^{-1})	LOD (cfu.mL^{-1})	Ref.
1.	rGO passivated with an ultrathin layer of Al_2O_3	FET	10^3 to 10^5	–	[28]
2.	Thin active layers of reduced graphene oxide/polyethylenimie	EIS	1×10^1 to 1×10^4	10	[5]
3.	IDAM coupled with magnetic nanoparticle-antibody conjugate	EIS	–	7.4×10^3	[29]
4.	Quartz crystal Au electrode/protein A/Abs	EIS	1.6×10^3 to 1.6×10^6	10^3	[30]
5.	AuNPs/Abs	EIS	1.5×10^2 to 1.5×10^7	1.5×10^2	[31]
6.	SPCE/graphene/AuNPs/Abs	EIS	1.5×10^3 to 1.5×10^7	1.5×10^3	[32]
7.	Gold nanoparticles modified gold electrode	EIS	up to 10^7	48	[33]
8.	Nanocomposite encapsulating Platinum nanoparticles	Hydrogen detector using electro-chemical method	10 to 10^4	10	[34]
9.	Au electrode/SAM of neutravidin/biotin-Abs	EIS	–	10	[35]
10.	Antibody immobilized/2-amino terephthalic acid functionalized graphene oxide (Ab/NH_2-GO/SPEs)	EIS	2.2×10^2–2.2×10^8	2	Present Work

FET Field Effect Transistor, *IDAM* Interdigitated array microelectrode, *Abs* Antibodies, *AuNPs* Gold nanoparticles, *SPCE* Screen printed carbon electrode

electrodes yielded almost similar EIS responses. The RSD (relative standard deviation) computed between their R_{ct} values was 3.45%. This indicated about a satisfactory reproducibility of the Ab/NH_2-GO/SPEs. The long-term stability of the bioelectrodes (stored at 4 °C) was also studied. As their EIS responses recorded against a fix *E. coli* concentration highlight, the Ab/NH_2-GO/SPEs sensor were fairly stable for at least up to 60 days (Fig. S5).

The specificity of the present Ab/NH_2-GO/SPEs has been evaluated against some other potentially interfering bacteria, e.g., *S. arlettae* and *S. aureus* (Fig. 2d). Only the target bacterium could induce a distinguished response. The base R_{ct} value of Ab/NH_2-GO/SPEs did not show any change toward non-specific bacteria, suggesting a desired specificity of the Ab/NH_2-GO/SPEs.

A comparison between the key performance parameters of the Ab/NH_2-GO/SPE biosensor with previously reported electrochemical biosensors for *E. coli* is summarized in Table 1. Based on this available information, it can be claimed that the Ab/NH_2-GO/SPE is an efficient and possibly better sensing platform than many of the previously reported options. It can be outlined that the reported system can provide a rapid and sensitive analysis of *E.coli* in different samples.

Through the Ab/NH_2-GO/SPEs have offered highly sensitive detection of *E. coli*, it may be pertinent to identify limitations of the present system. The modification of electrode surfaces with graphene and graphene-derivatives is a challenging task. A large-scale production of graphene bioelectrodes may need the application of costly physical/chemical deposition methods. Pre-functionalized graphene has been used and its deposition on SPEs has been performed subsequently. However, suitable conditions will have to be optimized for the physical/chemical deposition of pre-functionalized graphene on the SPEs. At present, the Ab/NH_2-GO/SPEs can be projected as single shot or disposable assay; nonetheless, future studies may be taken up to regenerate the graphene based biosensing electrodes.

Conclusions

The present research work demonstrates that the modification of graphene oxide (GO) with 2-aminoterephthalic acid is a viable option for obtaining amine functionalized GO. The resulting material can be easily interfaced with the antibodies. Compared to its reduced form, i.e. rGO, the option of using GO is considered a better approach as far as device development is concerned. Electrochemical impedance spectroscopy (EIS) as the signal collection technique nicely supplements the use of NH_2-GO based electrodes. It is easier to record frequency modulations than other types of electrochemical signals, such as amperometric or voltametric.

The present method has discussed the role of 2-amino terephthalic acid modified GO as a sensing material for the sensitive and selective detection of *E. coli*. The same electrodes can be modified with biorecognition probes, such as enzymes, aptamers and DNAs to develop various other types of biosensors.

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Compliance with ethical standards

Competing interests The author(s) declare that they have no competing interests.

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