Electrochemical genosensor based on carboxylated graphene for detection of water borne pathogen

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Abstract

This work reports the application of newly synthesized carboxylated graphene nanoflakes (Cx-Gnfs). The Cx-Gnfs were synthesized by wet chemical method in sulfuric acid/nitric acid mixture and was further electrophoretically deposited on Indium Tin oxide (ITO) coated glass substrate using Mg^{2+} ions which provides an overall charge to the materials for deposition onto the anode. The materials were characterized using SEM, TEM, contact angle, UV-Visible spectroscopy, FT-IR, XRD and electrochemically characterized by cyclic voltammetry, chronocoulometry and electrochemical impedance spectroscopy. The sensitive quantitative determination of nucleic acid *Escherichia coli* O157: H7 (*E. coli*) has been done using Cx-Gnfs and r-GO as the sensing layer using electrochemical impedance spectroscopy. The electrochemical results reveal that the Cx-Gnfs based genosensor exhibits a linear response to complementary DNA (10^{-6} M- 10^{-17} M) with a detection limit of 1×10^{-17} M while the rGO based genosensor shows a detection limit of 1×10^{-15} M. Under optimal conditions, this Cx-Gnfs based genosensor was found to retain about 85% of its initial activity after being used for 6 times.

Keywords: E.coli, graphene nanoflakes, biosensors, reduced graphene oxide, electrophoretic deposition.

1. Introduction

Pathogenic bacteria are considered to be serious threat to human health mainly due to food borne and water borne illnesses [1, 2]. The consumption of less processed products such as fruits, vegetables and ready to eat foods are the major source of food borne outbreaks by this pathogen which has created a major concern for their immediate monitoring to ensure that the food products are safe for consumption. In spite of the availability of regulations and methods of good manufacturing practices, hazard analysis and critical control point which are working for keeping a check on the presence of *E.coli* in food it has not been possible to achieve the desired result.

The most commonly practiced method in the area of food and safety for detection and enumeration of *E. coli* O157:H7 are conventional techniques which are based on culture and molecule. The culture-based method includes enumeration of bacterial colonies by growing bacteria in nutrient rich medium. While the molecule based method of enumeration are done by polymerase chain reaction (PCR) technique which is highly specific and sensitive for the detection of specific sequences of nucleic acid of the targeted bacteria using the hybridization methods. The molecular method is known to be more rapid as compared to the conventional culture methods [3-5].

The conventional methods of culturing, PCR, flow cytometry, solid-phase cytometry and ATP bioluminescence etc are used in the public health laboratories all over the world for the detection of *E.coli* O157:H7. They are based on standard protocols. In spite of these advantages the conventional methods have some drawbacks such as need of bulky instrumentations, large amount of medium which creates biological waste, time for culture and enumeration, interference by other substances of food matrices, requirement of proper training for careful handling of the instrument and a certain level of expertise for interpretation of results [6-9]. Hence, there is a need for the development of method for rapid detection of *E.coli* with improved specificity, reliability, feasibility, miniaturization, cost of detection and enumeration. In this respect, biosensors are the one of the most helpful and applied devices for monitoring the chemical and biological analytes. The role of biosensor is sensitive, selective and rapid identification using specific recognition elements. Among various recognition elements the detection using nucleic acid is pivotal in various disciplines such as human health, food

industries, environmental monitoring, forensics, diagnosis of infection and mutations etc. [10-12].

With the advent of nanotechnology, the construction of biosensors has become more fascinating. The use of nanoscale material helps to achieve a direct wiring of electrode with the enzyme, provides a larger surface area for electrochemical reaction, and also creates a nano-encryption for biomaterials so that the signal of biological recognition element can be amplified. The nanostructured material such as nanoparticles, nanotubes, nanowires etc of carbon or other metal oxides are used for recognition of biological events in electrochemical biosensors due to their enhanced properties [13, 14]. In this respect, graphene based materials are very well known. Further, the functionalization and chemical processing of graphene with other groups has lead to its wider application in field of nanostructured materials. The functionalization of graphene is generally done at the edges or at their basal planes. In this context, we herein report an application of carboxylated graphene nanoflakes (designated further as Cx-Gnfs). A new protocol has been used for the synthesis of graphene nanoflakes in which graphene edges have been modified with carboxylate groups [15, 16].

Similar types of materials are also reported in literature for the development of *E.coli* biosensors [10-12, 17-27]. Many reports for the genosensing of *E.coli* using different nanomaterials are also available [10-12, 24-27]. A genosensor for *E.coli* detection based on GO/chitosan nanocomposite has been reported by Xu *et al.* [24]. A report by Pandey *et al.* which uses microstructural patterned dendrites and flowers of cysteine as a platform for the detection of *E.coli* in the range of 10^{-6} to 10^{-14} M and 10^{-6} to 10^{-15} M with their respective detection limit as 1×10^{-14} M and 1×10^{-15} M is available [10, 25]. Some other applications on DNA hybridization study of *E.coli* are also available using cationic poly(lactic-co-glycolic acid) (PLGA) microspheres encapsulating the iron oxide nanoparticles and the detection limit of the sensor was found to be 8.7×10^{-14} [11]. Our group has reported three different sensors for detection of *E.coli* out of which one is based on graphene oxide -iron oxide-chitosan hybrid nanocomposite (GIOCh) film [26], another one used graphene oxide-nickel ferrite-chitosan (GO/NiF/ch) nanocomposite [27] and third one used polypyrrole-GO decorated GNP film (GO-GNP/PPY), and respective detection limits were found to be 1×10^{-14} M, 1×10^{-16} M and 1×10^{-15} M [12]. The reported works of *E.coli* genosensor possesses flaws in terms of sensitivity, selectivity, detection time, portability and reusability of the sensor.

Here in this work we are reporting genosensor for *E.coli* O157:H7, using a newly reported material Cx-Gnfs using the electrodeposition mode of fabrication of electrode. The main purpose of choosing this material as the electrode modifier for the development of genosensor is due to its carboxylated groups which facilitate the targeted attachment of nucleic acid. The results were compared with those obtained on chemically reduced graphene oxide (r-GO) using technique of electrochemical impedance spectroscopy. The sensor performance of the Cx-Gnf was found to be better than r-GO and most of reported works [Table 1].

2. Chemicals and Material required:

Graphite powder, MWCNTs, 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS) of analytical grade were purchased from Sigma-Aldrich. The specific sequence of *E. coli* probe (17 mer.) has been identified from the 16 s rRNA coding region of the *E. coli* genome. The complementary, non-complementary and one-base mismatch target sequences are obtained from Sigma Aldrich, Milwaukee, USA. Sequences of oligonucleotide probes are listed as below:

Probe I: probe DNA (pDNA): amine-5'-GGT CCG CTT GCT CTC GC-3'.

Probe II: complementary DNA (cDNA): 5'-GCG AGA GCA AGC GGA CC-3'.

Probe III: non-complementary DNA: 5'-CTA GTC GTA TAG TAG GC-3'.

Probe IV: one-base mismatch DNA: 5'-GCG AGA GAA AGC GGA CC-3'.

The oligonucleotide solution is prepared in Tris-EDTA buffer (1 M Tris-HCl, 0.5 M EDTA) of pH 8.0 and is stored at -20° C prior to use.

3. Experimental Section

3.1 Synthesis of Cx-Gnfs

MWCNT (1.00 g, Bayer Materials Science) were dispersed in 100 mL of a 3:1vol% mixture of H₂SO₄ (97w/w) and HNO₃ (70w/w) *via* ultrasonication for 30 min. The mixture was heated at 100°C for 2 h, left to cool, and diluted three-fold with deionized water. The resulting black dispersion was filtered (0.2 µm Whatman track-etched polycarbonate membrane) to remove any unreacted carbon material on the membrane. Finally the black filtrate was neutralized by the addition of KOH pellets under external ice bath cooling. After neutralization a large amount of white precipitate (consisting of K₂SO₄ and some KNO₃) was formed, which was

separated from the black filtrate by filtration. The dispersion was re-acidified by the addition of dilute formic acid and then dialyzed against high purity *Milli-Q* deionized water using a pre-treated SpectraPor 3 regenerated cellulose dialysis membrane (MWCO 3.5 kDa, Spectrum laboratories). The dialysis procedure was considered complete when the conductivity of the water surrounding the membrane decreased below $5\mu\text{S}\cdot\text{cm}^{-1}$, which was determined using a Mettler Toledo conductivity meter using a Mettler Toledo LE703 conductivity sensor. The pure brown-black dispersion underwent lyophilisation to obtain 222 mg of a pure brown-black material, identified as Cx-GNFs [15].

3.2 Synthesis of red-GO

The reduction of graphene oxide (GO) has been conducted using the two-step reduction procedure. Upon dispersion in deionized water, the pH of the solution is increased to 10 using 5 wt% sodium carbonate solution. Sodium borohydride (800 mg) was directly added to a 400 mL GO suspension (1 mg mL^{-1}) under magnetic stirring, and the mixture was kept at 80°C for 1 h with constant stirring, leading to a change in the color from brown to black. The solid product was then purified with water and washed with ethanol, followed by drying and re-dispersed in H_2SO_4 at 120°C . This solution is again re-filtered through Millipore filter paper to yield reduced graphene oxide (r-GO) [29].

3.3 Fabrication of electrodes:

For electrophoretic deposition a very well dispersed stock solution of Cx-Gnfs (20 mg dL^{-1}) in acetonitrile was prepared by ultrasonication (80 W, 0.25 A) for about 2 h. Then, $100\mu\text{L}$ of this stock solution was dispersed in 10 mL of acetonitrile to make the colloidal suspension of Cx-Gnfs. The film was electrophoretically deposited using two-electrode cell by applying a DC voltage (140V, 2 min), where platinum foil was used as the cathode and a precleaned ITO-coated glass substrate as anode. To create surface charge on the Cx-Gnfs, 10^{-5} mol of $\text{Mg}(\text{NO}_3)_2\cdot 6\text{H}_2\text{O}$ was added into the suspension as electrolyte for EPD [30]. The deposited film (Cx-Gnfs/ITO electrode) was then removed from the suspension followed by washing with deionized water and drying. The schematic view of electrodeposition and fabrication of electrode has been illustrated in fig. 1.

Fig. 1 Schematic representation of (A) fabrication of electrode based on graphene materials and (B) electrochemical genosensing of E.coli using the fabricated electrode.

4. Result and Discussion

4.1 Physical Characterization

4.1.1 SEM studies:

SEM studies were carried out to study the surface morphology of the electrodeposited nanomaterial on ITO electrode. Fig. 2 (A) reveals the surface of ITO after electrophoretic deposition of Cx-Gnfs nanoflakes. A uniform deposition of nanoflakes was observed on the surface of ITO electrode [*cf.* Fig. 2(A)]. The inset to fig. 2 (A) clearly reveals that the flaky structures of the nanomaterial have been lost and the nanoflakes are aggregated to form cauliflower structures of uniform size. The fig. 2 (B) shows the SEM image of r-GO after electrodeposition on the ITO electrode. From the figure the uneven electrodeposition of large nanostructures on the surface of ITO can be observed. The large structure might be due to flake-like crumpled glossy sheets of r-GO which is further confirmed at higher magnification [*cf.* inset of the fig. 2 (B)].

Fig. 2 SEM image showing the morphology of the surface after electrodeposition of (A) Cx-Gnfs and (B) r-GO on the ITO electrode.

4.1.2 TEM studies:

The microstructure and crystal pattern of the nanomaterial was understood using TEM images. The selected area electron diffraction (SAED) pattern obtained is helpful in providing information for the nature of the sample. Fig. 3 (A) depicts the nano-flakey structure of Cx-Gnfs having an average diameter of 50 nm [15]. The SAED pattern of Cx-Gnfs, shows the rings of the pattern possesses bright spots which is due to the polycrystalline nature of the nanomaterial [*cf.* inset Fig. 3(A)]. Crumbled and agglomerated but well exfoliated sheets of r-GO can be observed from fig. 3 (B). The inset to the Fig. 3(B) illustrated a SAED pattern of r-GO having six-fold symmetric pattern owing to the crystalline nature of the nanomaterial.

Fig. 3 TEM showing the nanostructures (A) Cx-Gnfs and (B) r-GO with the respective inset showing the SAED pattern of the nanomaterials.

4.1.3 Contact Angle studies:

Contact angle (CA) measurements carried out using the sessile drop method shows that after the electrodeposition of Cx-Gnfs on ITO electrode there was a decrease in the CA from 85.4° [*cf.* suppl. material fig. S1 image (i)] to 44.8° [image (ii)]. The decrease in CA may be due to the introduction of Cx-Gnfs having polar groups $-\text{COO}^-$ which increases the hydrophilicity of the electrode surface. A further decrease [*cf.* suppl. material fig. S1 image (iii)] in CA (22.52°) after the immobilization of pDNA onto Cx-Gnfs/ITO electrode was observed which may be due

to enhancement of hydrophilicity caused by the presence of negatively charged phosphodiester backbone of DNA.

4.1.4 UV-Visible studies:

The UV-visible spectroscopic studies for Cx-Gnfs and r-GO were carried out in the wavelength range of 200-800nm as indicated in suppl. material fig. S2, curve (a) and curve (b) respectively. There was a monotonic increase in the absorbance from 500 nm in Cx-Gnfs and from 350 nm in r-GO. This might be due to π - π^* transition of C-C aromatic bonds of both the carbon nano-materials. The band gap of Cx-Gnfs was found to be 2.4 eV which is lower than that of r-GO (3.5 eV) using the Tauc equation. The difference in band gaps might be due to non-availability of functional groups on the basal plane of Cx-Gnfs as compared to the oxygen functionalities present in the r-GO [15, 29].

4.1.5 FT-IR analysis:

FTIR analysis of Cx-Gnfs shows an intense peak at 1722 cm^{-1} that signifies the presence of C=O group [*cf.* suppl. material fig. S3 (A)], while the band at 3400 cm^{-1} is due to O-H stretching vibration. The bands seen at 1710 and 1410 cm^{-1} are due to C=O stretching and O-H bending vibrations of the carboxyl group present in r-GO. The band appearing at 1585 cm^{-1} is due to a C=C stretching mode while the band observed at 1207 cm^{-1} is due to C-OH stretching vibration. C-H stretching and bending vibrations are observed at 2950 and 866 cm^{-1} , respectively. A broad band found at 3400 cm^{-1} is due to O-H stretching vibration of the hydroxyl group present in the r-GO [*cf.* suppl. material Fig. S3 (B)].

4.1.6 XRD analysis:

The XRD data is an important tool in the characterization of the materials by giving an idea about interlayer spacing and its corresponding diffraction angle. The XRD of Cx-Gnfs and r-GO is presented in fig. 4(A) and 4(B) respectively. The characteristic broad peaks at 22.9° and 40.4° in Cx-Gnfs can be corroborated with the data of carboxyl (COOH) functionalized MWCNTs (Since MWCNTs are used as starting material in the synthesis of Cx-Gnfs) [31] and a broad reflection between 25° - 30° can suggest the formation of disordered graphitic structure as observed in reduced graphene. The fig. 4(B) shows a broad reflection in the range of 21.6° - 36.8°

in the case of r-GO which may be due to the reduction of GO by sodium borohydride which causes a shift in diffraction angle of GO (10.2°) suggesting the poor arrangement of the layers[32].

Fig. 4 XRD pattern of (A) Cx-Gnfs and (B) r-GO.

4.2 Electrochemical characterizations:

4.2.1 Cyclic Voltammetry

The electrocatalytic behavior of the Cx-Gnfs was compared to that of the r-GO using the cyclic voltammetry technique in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl). The cyclic voltammetry provided an insight in the electron transfer mechanism. The mechanism for the electron transfer by any electrode material can be best explained by the heterogeneous rate constant. This rate constant further depends upon another parameter of electron property named as the density of electronic state, a high value of which signifies that the electron of correct energy is available for transfer from the electrode to redox species [33, 34].

Various kinetic parameters were calculated using cyclic voltammetry techniques for the electrophoretically deposited material namely Cx-Gnfs and r-GO on ITO. The fig. 5 (A) depicts the voltammogram of (a) bare ITO, (b) Cx-Gnfs/ITO and (c) r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl). There is an increase in the redox peak current on Cx-

Gnfs/ITO and r-GO/ITO as compared to that of bare ITO [*cf.* fig. 5 (A) curve (a)] which might be due to the fast electron transfer kinetics at the carbon materials modified electrodes suggesting the carbon based nanomaterial provides an electrical wiring between the electrode and the redox active species (here $[\text{Fe}(\text{CN})_6]^{3-/4-}$). The electrocatalytic performance of the Cx-Gnfs/ITO [*cf.* fig. 5 (A) curve (b)] towards the redox species is found to be low as compared to r-GO/ITO [*cf.* fig. 5 (A) curve (c)]. The plausible reason of low catalytic nature of Cx-Gnfs is the presence of negatively charged carboxylate groups on the edges of the Cx-Gnfs which might create a hinderance in electron transfer between electrode and $[\text{Fe}(\text{CN})_6]^{3-/4-}$. Besides this the peak currents [both anodic (I_{pa}) and cathodic (I_{pc})] is found to vary linearly with the square root of scan rate ($v^{1/2}$) depicting the process to be diffusion limited. The fig. 5(B) denotes the linear variation of peak current with $v^{1/2}$ for Cx-Gnfs/ITO [plot (a)] and r-GO/ITO [plot (b)] in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl). The corresponding linear equation (1) and (2) is for Cx-Gnfs/ITO and (3) and (4) is for r-GO/ITO respectively.

$$I_{\text{pa}}/A = 4.5430 v/Vs^{-1} + 0.1651 \quad [1] \quad R^2 = 0.997$$

$$I_{\text{pc}}/A = -3.7210 v/Vs^{-1} - 0.3070 \quad [2] \quad R^2 = 0.993$$

$$I_{\text{pa}}/A = 8.200 v/Vs^{-1} + 0.0309 \quad [3] \quad R^2 = 0.997$$

$$I_{\text{pc}}/A = -5.3331 v/Vs^{-1} - 0.4060 \quad [4] \quad R^2 = 0.991$$

The Fig. 5 (C) and 5 (D) corresponds to the Laviron plot (plot between peak potentials and $\log v$) of Cx-Gnfs/ITO and r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl) respectively. The linear equations for the linear part of Laviron plot for Cx-Gnfs/ITO are eqs. (5) and (6) and for r-GO/ITO and r-GO/ITO eqs. (7) and (8) depicted below:

$$E_{\text{pa}}/V = 0.4849/V + 0.2050 \log v \quad [5] \quad R^2 = 0.998$$

$$E_{\text{pc}}/V = -0.1410/V - 0.1993 \log v \quad [6] \quad R^2 = 0.992$$

$$E_{\text{pa}}/V = 0.4973/V + 0.2131 \log v \quad [7] \quad R^2 = 0.999$$

$$E_{pc}/V = -0.1536/V - 0.1174 \log v \quad [8] \quad R^2 = 0.998$$

The kinetic parameters such as α_{ec} (electron transfer co-efficient) and k_{avg} (heterogenous rate constant) were calculated using the Laviron equations (9) and (10) [35, 36];

$$\alpha_{ec} = S_{pa}/S_{pa} + S_{pc} \quad [9]$$

$$K_{avg} = \frac{nF\alpha_{ec}v_c}{RT} = \frac{nF(1-\alpha_{ec})v_a}{RT} \quad [10]$$

Where S_{pa} and S_{pc} are the anodic and cathode slopes of lavron plot respectively, v_a and v_c are the respective scan rates. The α_{ec} and k_{avg} value for Cx-Gnfs/ITO are 0.50 and 3.89 s^{-1} while that of r-GO/ITO are 0.64 and 2.80 s^{-1} in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl).

The surface coverage of the electrodeposited Cx-Gnfs and r-GO on ITO were also determined using another Laviron equation (11) [37];

$$I_p = \frac{n^2 F^2 A \Gamma v}{4RT} \quad [11]$$

The anodic and cathodic average of Γ value for Cx-Gnfs/ITO is found to be $9.25 \times 10^{-8} \text{ molcm}^{-2}$ and for r-GO/ITO is $0.21 \times 10^{-8} \text{ molcm}^{-2}$.

Based on the comparison of the results of both the electrode materials it can be clearly inferred that Cx-Gnfs shows a better electron tranfer kinetics. The electrodeposited Cx-Gnfs on ITO will be able to provide a larger surface area for immobilization of the probe DNA segment as compared to r-GO on ITO.

Fig. 5 (A) Electrocatalytic response of (a) bare ITO, (b) Cx-Gnfs/ITO and (c) r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl), (B) plot between current and $v^{1/2}$, (C) Laviron Plot for Cx-Gnfs/ITO and (D) Laviron plot for r-GO/ITO.

4.2.2 Chronocoulometry

The charge can be obtained by integration of current with respect to time that is the response of the chronocoulometry experiment [38]. The relation of the charge (Q) with time (t) can be given by Anson's equation (12) obtained by integrating the Cottrell equation;

$$Q = \frac{2nFAD^{1/2}C_0t^{1/2}}{\pi^{1/2}} \quad [12]$$

Where, Q, n, A, D, C₀, and t stands for charge, number of electron transferred, area of the electrode (here 0.22 cm²), diffusion coefficient, bulk concentration and time respectively. The fig. 6(A) shows the forward scan of the choronocoulometric response of (a) bare ITO, (b) Cx-Gnfs/ITO, and (c) r-GO/ITO in 5 mM [Fe(CN)₆]^{3-/4-} (in PBS, 100 mM, pH 7.0, 0.9% NaCl). Whereas plots in fig. 6(B) shows the linear dependence of Q on t^{1/2}, called as Anson plot. The slopes of the Anson plot is used for calculating the diffusion co-efficient using above equation (12) which will be further helpful in explaining the electron transfer kinetics of both the materials (Cx-Gnfs and r-GO). The diffusion coefficient (D) for Cx-Gnfs/ITO and r-GO/ITO were found to be 4.79 x 10⁻¹⁰ cm²s⁻¹ and 4.12 x 10⁻¹⁰ cm²s⁻¹ respectively. The higher value of the D for Cx-Gnfs/ITO further corroborates the better electron transfer kinetics of the material as compared to that of r-GO.

4.2.3 Electrochemical impedance spectroscopy (EIS)

EIS is a widely used electrochemical technique, due to its fast and sensitive response occurring at the electrode-electrolyte interface. EIS consist of a semicircle portion, observed at higher frequencies that corresponds to the electron transfer limited process, followed by a linear part characteristic of the lower frequency attributed to a diffusion limited electron transfer. The semicircle diameter of EIS spectra gives value of charge transfer resistance (R_{ct}) [26, 38]. The increase in the diameter of the semicircle reflects increase in the R_{ct} and the numerical values of R_{CT} can be derived from experimental impedance spectra by fitting an equivalent circuit model based on modified Randles and Ershler model to the data. To investigate the electrochemical response of the fabricated pDNA/Cx-Gnfs/ITO bioelectrode EIS technique has been adopted. As the oligonucleotide chain is composed of amine rich nucleoside residues, it is expected that the presence of free functional groups on the Cx-Gnfs/ITO surface may lead to chemisorption of the

complementary targets thereby providing false positive signal. Hence, to avoid the competition of hybridization event with non-specific adsorption of the cDNA, a study for optimization of the pDNA concentration has been performed. As shown in suppl. material fig. S4 (A) by increasing the amount of pDNA from 10^{-10} M to 10^{-4} M, the electrochemical signal is levelled off at 10^{-6} M. So, it was presumed that the maximum immobilization was achieved at this concentration (10^{-6} M).

To understand the hybridization process the analytical performance of the pDNA/Cx-Gnfs/ITO bioelectrode towards recognition of the target oligonucleotides has also been optimised. The EIS response of the pDNA/Cx-Gnfs/ITO bioelectrode in the presence of 1 μ M complementary target DNA at different incubation time (5-25 min) was conducted. It was observed that the R_{ct} value increases with increase in the incubation time upto 15 min [*cf.* suppl. material fig. S4 (B)], showing that the extent of hybridization is accomplished within this duration.

The change in R_{ct} value after each modification step was analysed using phosphate buffer (PBS, 100 mM, pH 7.0, 0.9% NaCl) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as the redox probe. Under the optimum conditions, the bare ITO electrode shows the R_{ct} value of 1.9 K Ω (curve not shown). After electrophoretic deposition of Cx-Gnfs [*cf.* Fig. 6(C); curve (i)], there was a decrease in the R_{ct} value (1.2 K Ω). This decrease in the R_{CT} value clearly indicates that there was a significant change in the reactivity after deposition of Cx-Gnfs which might be due to structural change that facilitates strong adsorption leading to faster electron transfer kinetics. Under similar condition the R_{CT} value obtained for the rGO/ITO electrode [*cf.* Fig. 6 (D); curve (i)] was 0.8 K Ω , which was lower, compared to that of Cx-Gnfs/ITO electrode. This change in the R_{ct} value is due to the good conductivity of rGO compared to Cx-Gnfs. Further, covalent binding of *E. coli* pDNA on Cx-Gnfs/ITO electrode [*cf.* Fig. 6 (C); curve (ii)] and r-GO/ITO electrode [*cf.* Fig. 6 (D); curve (ii)] results in enhancement of R_{ct} in each case which is due to the negatively charged phosphate backbone of pDNA exposed to the electrolyte, which in turn repel $[\text{Fe}(\text{CN})_6]^{3-/4-}$. When the pDNA is incubated with its complementary target DNA sequence (cDNA), the hybridization reaction occurs and more negatively charged phosphate backbones are introduced. Hence, the R_{ct} value further increases following the formation of double-stranded DNA [*cf.* Fig. 6 (C) and 6 (D); curve (iii)]. It can thus be inferred that with a change in negative charge and conformation transition, there is a change in the interfacial properties.

Fig. 6 (A) Chronocoulometric response of (a) bare ITO, (b) Cx-Gnfs/ITO and (c) r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (in PBS, 100 mM, pH 7.0, 0.9% NaCl), (B) Anson's Plot showing linear dependency of Q and $t^{1/2}$. EIS characterization of materials (C) (i) Cx-Gnfs/ITO, (ii) p-DNA/Cx-Gnfs/ITO and (iii) cDNA/pDNA/Cx-Gnfs/ITO and (D) (i) r-GO/ITO, (ii) p-DNA/r-GO/ITO and (iii) cDNA/pDNA/r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (PBS, 100 mM, pH 7.0, 0.9% NaCl).

4.3 Electrochemical response studies:

EIS was used for the quantitative determination of *E. coli*, as it provided an instant response, showing the efficiency of electron transfer between the electrode surface and the electro-active substances. The difference between the value of R_{ct} for the pDNA immobilized electrode after hybridization with cDNA ($\Delta R_{ct} = R_{ct} \text{ cDNA} - R_{ct} \text{ pDNA}$) has been used as the measurement signal. The EIS response of pDNA/Cx-Gnfs/ITO bioelectrode towards the hybridization of cDNA can be seen from the fig. 7(A). The analytical signal for pDNA/Cx-Gnfs/ITO bioelectrode (in terms of ΔR_{ct}) shows linear relationship with the logarithmic value of the complementary target DNA concentration ranging from 10^{-17} to 10^{-6} M [cf. Fig. 7(B)] and follows the following equation:

$$\Delta R_{ct} = 7981.90 + 462.25 [\log (\text{concentration of cDNA})] \quad [13] \quad R^2 = 0.995$$

The detection limit is calculated to be 1×10^{-17} M using the expression $3\sigma/\text{sensitivity}$, where σ is the standard deviation of the blank electrode. The response of pDNA/rGO/ITO bioelectrode towards hybridization with cDNA can be observed from fig. 7 (C) and similar linear relationship with the logarithmic value of the complementary target DNA concentration for pDNA/rGO/ITO bioelectrode was obtained in the range from 10^{-15} to 10^{-6} M [cf. Fig. no. 7 (D)] which follows the following equation:

$$\Delta R_{ct} = 2420.36 + 170.33 [\log (\text{concentration of cDNA})] \quad [14] \quad R^2 = 0.995$$

Whereas the detection limit for pDNA/rGO/ITO bioelectrode was calculated to be 1×10^{-15} M by using the expression $3\sigma/\text{sensitivity}$.

The detection limit obtained for pDNA/Cx-Gnfs/ITO bioelectrode is better as compared to that obtained using pDNA/rGO/ITO bioelectrode, which might be attributed due to the presence of more functional group on the surface of Cx-Gnfs compared to rGO. These functional groups provide a conducive environment for the better immobilization of pDNA on Cx-Gnfs/ITO electrode.

Fig. 7 (A) EIS data for detection of cDNA on pDNA immobilized on electrodeposited Cx-Gnfs/ITO electrode, (B) the calibration plot detection of c-DNA on pDNA/Cx-Gnfs/ITO, detection of c-DNA on p-DNA immobilized on electrodeposited (C) r-GO/ITO electrode and (D) the calibration plot detection of cDNA on pDNA/r-GO/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (PBS, 100 mM, pH 7.0, 0.9% NaCl)

4.4 Selectivity study

The specificity of the pDNA/Cx-Gnfs/ITO bioelectrode towards different target DNA sequences (non-complementary, one base mismatch and complementary) has been studied using EIS [suppl. material fig. S5]. After incubation of the bioelectrode with non-complementary DNA [*cf.* suppl. material fig. S5 curve (ii)], there was a slight or negligible change in the R_{ct} value with respect to the pDNA (curve i). Since, the non-complementary DNA bases do not match with the pDNA bases it is expected that there is no hybridization taking place and hence the R_{ct} should result in a semicircle diameter similar to that of pDNA/Cx-Gnfs/ITO bioelectrode. When the pDNA/Cx-Gnfs /ITO bioelectrode was immobilized with one base mis-match DNA sequence there was a slight increase in semicircle diameter in comparison to that of the pDNA/Cx-Gnfs/ITO bioelectrode (curve iii). This explains the partial hybridization of pDNA with the complementary bases of the one base mis-match DNA resulting in increased negative charge of the electrode surface which repels the marker ion. These results reveal the selectivity of the pDNA/ Cx-Gnfs /ITO bioelectrode towards different target DNA sequences. The negligible change in the value of R_{ct} has been observed when pDNA/Cx-Gnfs/ITO bioelectrode was treated with non-complementary DNA and one-base mismatch, which shows that fabricated bioelectrode is highly specific for *E. coli* detection.

4.5 Reusability and stability of the bioelectrode

The fabricated sensor seems to be stable enough to allow the regeneration of pDNA/Cx-Gnfs/ITO bioelectrode after binding of cDNA. The removal of the cDNA is done by thermal denaturation in which the used electrodes are immersed in a mixture solution containing Tris buffer-HCl (10mM) with pH 8.0 and 1mM EDTA at 100° C for 5 min, further taking it to the low temperature using an ice bath for 30 min. There was decrease in the R_{CT} value after each regeneration step and an average loss of ~15 % is noted after 6 cycle of regeneration [*cf.* suppl. material fig. S6 (A)]. The decrease in the signal might be observed due to the surface fouling of the bioelectrode after every regeneration process. The storage stability of fabricated bioelectrode was investigated by recording five times by measuring the signal every week over a continuum of 30 days. The fabricated bioelectrode showed as decrease in the signal response of about ~18 % (stored at 4° C) [*cf.* suppl. material fig. S6 (B)]. The sensor performance of the Cx-Gnf was

found to be better than r-GO and most of reported works [Table 1] indicating the suitability of prepared material.

Conclusions:

Herein we report a highly sensitive nucleic acid sensor for the determination of *E.coli* using a newly synthesized carbon material (Cx-Gnfs). The electrophoretic method of deposition of material was used to fabricate the working electrode and further the material was characterized using physical methods such as SEM, TEM, contact angle, UV-Visible spectroscopy, FT-IR, XRD and electrochemically using cyclic voltammetry, chronocoulometry and EIS. The application of the proposed electrode was done for the genosing of *E.coli* nucleic acid strand using EIS technique. It was observed that the deposited nanomaterial enhanced DNA detection and better sensitivity, selectivity, detection limit and reproducibility were obtained. The efforts are in progress for the development of technology for *E.coli* monitoring.

Conflict of Interest

There authors have no conflict of interest.

Acknowledgements

N. Jaiswal is thankful to UGC, New Delhi for providing fellowship and CSIR, India for awarding SRF. C.M. Pandey acknowledges the financial support received from Department of Science and Technology, India under DST-INSPIRE Faculty project (Grant No. DST/INSPIRE/04/2015/000932). The authors are sincerely thankful to Prof. O.N Srivastava (Dept. of Physics, I.Sc. B.H.U) and Prof. Rajiv Prakash (IIT B.H.U) for providing TEM and SEM facility respectively.

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Table 1: Comparison of result with the previously reported work of *E.coli* genosensor

S.No	Working electrode	Detection range	Limit of detection	References
1.	CysFI/Au bioelectrode	1.0×10^{-6} M to 1.0×10^{-15} M	1.0×10^{-15} M.	[10]
2.	GO-GNP/PPY	1.0×10^{-6} M to 1.0×10^{-15} M	1.0×10^{-15} M	[12]
3.	GO/CS/GCE	1.0×10^{-14} to 1.0×10^{-8} M	3.58×10^{-15} M	[24]
4.	denCys/Au	1.0×10^{-14} to 1.0×10^{-8} M	1.0×10^{-14} M	[25]
5.	GIOCh/ITO	10^{-14} to 10^{-6} M	1×10^{-14} M	[26]
6.	GO/NiF/ch	10^{-16} to 10^{-6} M	1×10^{-16} M	[27]
7.	Cx-Gnfs/ITO	10^{-6} M to 10^{-17} M	1×10^{-17} M	Present work

GO/CS/GCE-graphene oxide-chitosan modified glassy carbon electrode, CysFI/Au bioelectrode-flowers of cystine self assembled on gold electrode, GO-GNP/PPY- polypyrrole-Graphene oxide decorated Gold nanoparticles, denCys/Au- dendritic cystine on gold electrode, GIOCh /ITO-graphene oxide modified iron oxide–chitosan hybrid nanocomposite immobilized on indium tin oxide electrode, GO/NiF/ch- graphene oxide–nickel ferrite–chitosan nanocomposite, Cx-Gnfs/ITO-Electrophoretically deposited carboxylated graphene nanoflakes on Indium Tin oxide glass substrate.

Supplementary Information

Electrochemical genosensor based on carboxylated graphene for detection of water borne pathogen

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Figure Captions:

Fig. S1 Contact angle measurements of (A) ITO electrode, (B) electrodeposited Cx-Gnfs/ITO electrode and (C) p-DNA/Cx-Gnfs/ITO

Fig. S2 UV-visible spectra of (a) Cx-Gnfs and (b) r-GO

Fig. S3 FT-IR spectra of (A) Cx-Gnfs and (B) r-GO

Fig. S4 Graph showing (A) the variation of the R_{ct} with change in pDNA concentration (B) the hybridization time for the cDNA.

Fig. S5 (A) Nyquist plots for the EIS measurements of (i) cDNA/pDNA/Cx-Gnfs/ITO, (ii) one base mismatch p-DNA/Cx-Gnfs/ITO, and (iii) non complementary p-DNA/Cx-Gnfs/ITO in 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ (PBS, 100 mM, pH 7.0, 0.9% NaCl).

Fig. S6 (A) Graph showing percentage change in the R_{ct} of the fabricated pDNA/Cx-Gnfs/ITO bioelectrode towards *E. coli* detection was determined after each regeneration cycle (6 cycles). (B) Bar diagram showing the stability of the fabricated pDNA/Cx-Gnfs/ITO bioelectrode.

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6