Supplementary file

Development of Highly Sensitive Label-Free Electrochemical Immunosensor for p16INK4a Detection: A Step Toward Early Cervical Cancer Diagnosis

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Experimental Section

Chemicals

All the chemicals dispensed were of analytical grade unless otherwise stated. N-hydroxysuccinimide (NHS), cystamine, gluteraldehyde, tetrachloroauric (III) acid trihydrate (99%, $HAuCl_4.3H_2O$) and bovine serum albumin (BSA, 96 %) were purchased from Sigma Aldrich, India. Ethanol (C_2H_5OH, 99 %), $K_3[Fe(CN)_6]^{3-}$, $K_4[Fe(CN)_6]^{4-}$, potassium chloride (KCl) and PBS tablet (pH- 7.4) were purchased from HiMedia. p16INK4a ELISA Kit (LS-F11076), Carcinoembryonic Antigen Protein (LS-G11700) were bought from LSBio. Recombinant CA125 (5609-MU-050) was bought from R&D systems. Recombinant carbohydrate antigen 19-9 (CA 19-9) (MBS2011087), SCCA recombinant protein (MBS313447) and MCM5 protein (MBS8533422) were bought from MyBioSource. All solutions were prepared using Millipore water (Millipore Sigma STMSV00US) or according to the instruction manual given by the respective company.

Instrumentation

Field Emission-Scanning Electron Microscopy (FE-SEM): Ultrahigh-resolution FE-SEM (JEOL JSM-6500F) instrument was using done by characterization of morphology of all materials. All samples were deposited onto the indium tin oxide (ITO) glass with the optimal procedure.

X-ray diffraction (XRD): PANalytical X'Pert PRO diffractometer with **the Cu Kα1 radiation (λ** = 1.540598 **nm) and** was used to measure the intensity at the 2**θ** range between 15 º and 75º with a step of 0.017° for all the analyses.

X-ray Photoelectron Spectroscopy (XPS): XPS study were analysis with an ESCA chemical analysis electron spectrometer (JEOL, JPS-9030) and vacuum multichamper systemusing a monochromattic X-ray source (Al Kα) radiation accompanied by a beam size of 10 mm was maintained for the measurements.

Electrochemical study

SP150 Bio-Logic Science Instrument (electrochemical workstation, France) was used for electrochemical analysis. The electrochemical cell involved of a three-electrode system with a glassy carbon electrode (GCE). GCE was a working electrode, a saturated Ag/AgCl electrode, and platinum (Pt) wire as a counter electrode. CV, EIS, and SWV were performed in the presence of 0.1 M KCl/10 mM $[Fe(CN)_6]^{3-/4-}$ (pH 7.4, 0.1 PBS,) mixture as a redox probe.

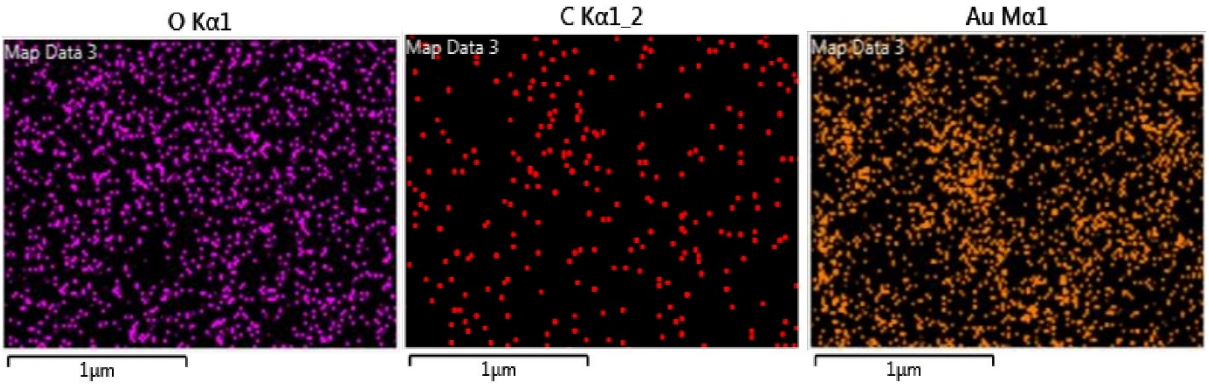

Figure S1: Energy dispersive X-ray (EDX) analysis

Clinical sample collection

Clinical serum samples were collected from Chittaranjan National Cancer Institute, Kolkata. The samples included ten individuals diagnosed with cervical cancer and five healthy participants. The study protocol was reviewed and approved by the Ethics Committee of the National Institute of Technology, Arunachal Pradesh, under the approval number NIT/AP/R&D/IEC/2021/21. For sample preparation, each serum sample underwent centrifugation at 3,000 rpm for 5 minutes at 4°C, allowing for the separation of the supernatant. The resulting supernatants were then carefully collected and stored at -80°C to preserve their integrity for subsequent analysis.

Optimization of the immunosensor

Glutaraldehyde incubation time

To optimize the incubation time of glutaraldehyde, cystamine modified electrode immersed in 2.5% glutaraldehyde solution for various time duration (0-90 minutes) at interval of 10 minutes and Square Wave Voltammetry (SWV) was used to measure the current. **Figure S2** illustrates that the resulting peak current decreased proportionally with an increase time. The peak current was almost steady after 70 min incubation which concludes optimization has been done completely due to glutaraldehyde saturation on the electrode. As a result, 70 min incubation time was selected for the immunosensor.

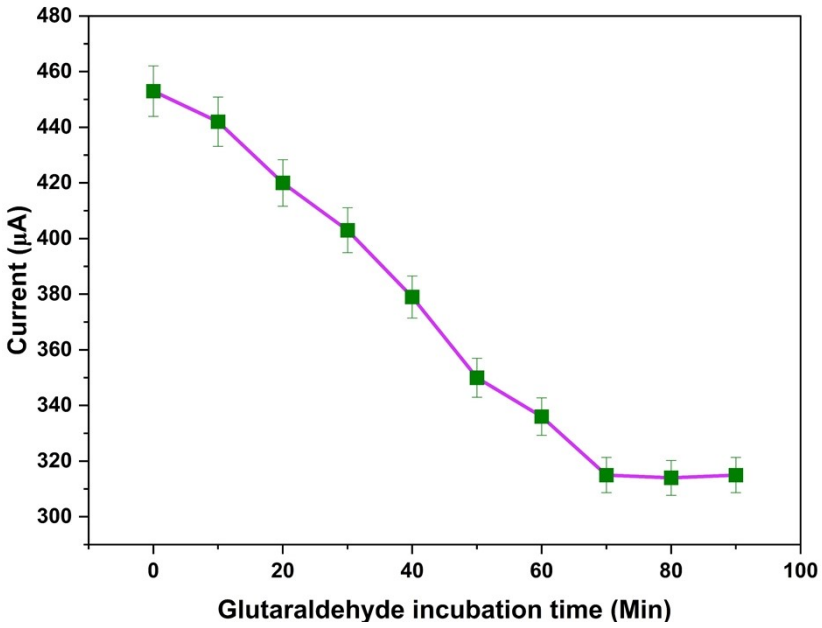

Figure S2. Glutaraldehyde incubation time optimization in 0.1M KCl/10 mM [Fe (CN)6]$^{3-/4-}$.

Antibody incubation time

The effectiveness of the immunosensor depends on optimizing the incubation time of p16INK4a antibody. To do this, we incubated the modified electrode with 4µg mL^{-1} antibody solution and at 5 minutes interval (0 to 35 minutes) we measured current using SWV (refer to **Figure S3.A**). As incubation time spanning was increasing, the peak current was decreasing, indicated nonconductive binding on the modified electrode surface. Interestingly, after 25 minutes the peak current reaching a stable state, indicating ideal conditions. This led to the conclusion that 25 minutes is the optimal antibody incubation time.

Antibody concentration

To optimize particular antibody concentration, various concentrations of the antibody (2 to 16 µg mL^{-1}) were tested. Due to antibody immobilization on the electrode surface, the peak current decreased as the antibody concentration increased gradually (**Figure S3.B**). Then, as the antibody concentration increased further, a plateau in peak current was seen after 10 µg mL^{-1} concentration, signifying electrode saturation at a certain concentration. Based on these results, the ideal antibody concentration was 10 µg mL^{-1}. All experiments were done in room temperature.

BSA incubation time

After successful antibody immobilization, the electrode kept for incubation in 1% BSA solution at room temperature. With the interval of 5 minutes (0 to 40 minutes) we measured current

using SWV. **Figure S3.C** clearly describes the drop in SWV peak current that occurred within the first 30 minutes of incubation. After 30 minutes, the peak current remained constant, suggesting that the electrode surface had successfully immobilized the most BSA possible. Hence, it was shown that the best incubation time for immobilizing BSA was 30 minutes.

Immune reaction duration

To enhance sensor development, a thorough dataset was acquired by experimenting with various immunoreaction incubation times. Consequently, the modified electrode was used to incubate a 500 pg mL^{-1} p16INK4a protein at room temperature for periods varying from 0 to 35 minutes. Current was measured at 5-minute intervals. As shown in **Figure S3.D**, the SWV peak current decreased proportionally with increasing incubation time up to 25 minutes, suggesting that the immobilized antibody fully interacted with antigen molecules. The peak current stayed nearly constant even after extending the incubation period, proving that 25 minutes is the ideal time for the antigen-antibody response.

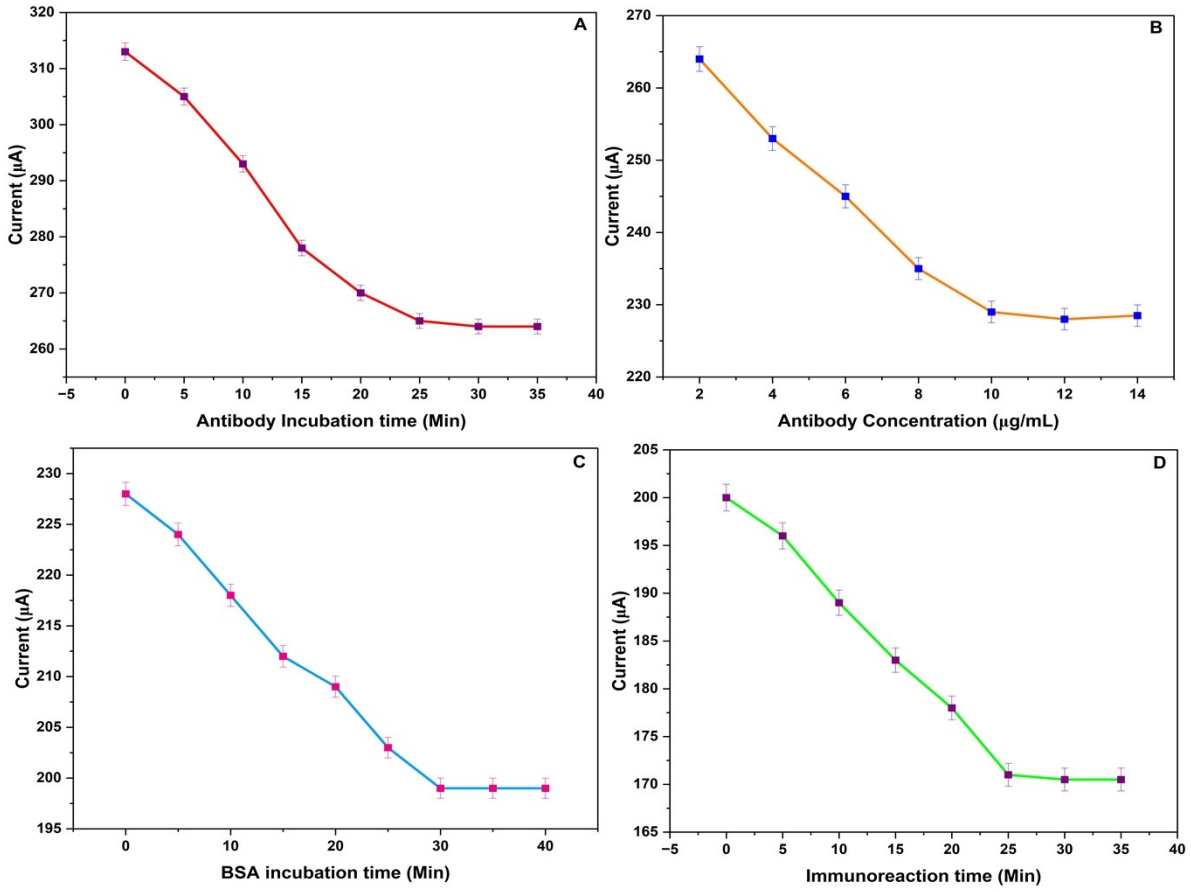

Figure S3. Optimization process for (A) Antibody incubation time; (B) Antibody concentration; (C) BSA incubation time; (D) Immunoreaction time in 0.1M KCl/10 mM [Fe (CN)6]$^{3-/4-}$.

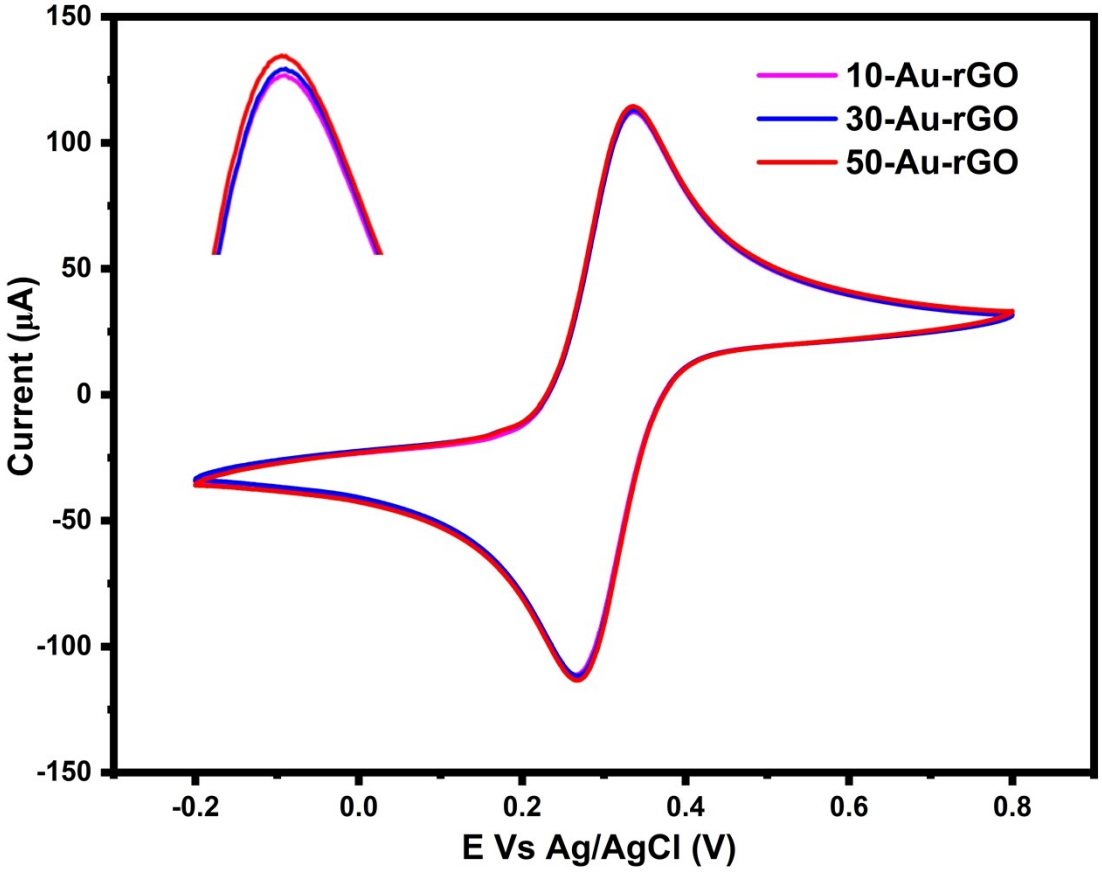

Figure S4. Cyclic voltammetry response of 10, 30, and 50 cycles of electro-deposition of Au/rGO hybrid film.