Functionalized Graphitic Carbon Nitride as an Efficient Electro-Analytical Platform for the Label-free Electrochemical Sensing of Interleukin-8 in Saliva Samples

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SUPPLEMENTARY INFORMATION

S1: Amino Acid Sequence of IL8 Protein

AKELRCQCIKTYSKPFHPKFIKELRVIESGPHCANTEIIVKLSDGREL CLDPKENWVQRVVEKFLKRAENS

S2: FT-IR spectra of modified electrodes at each step

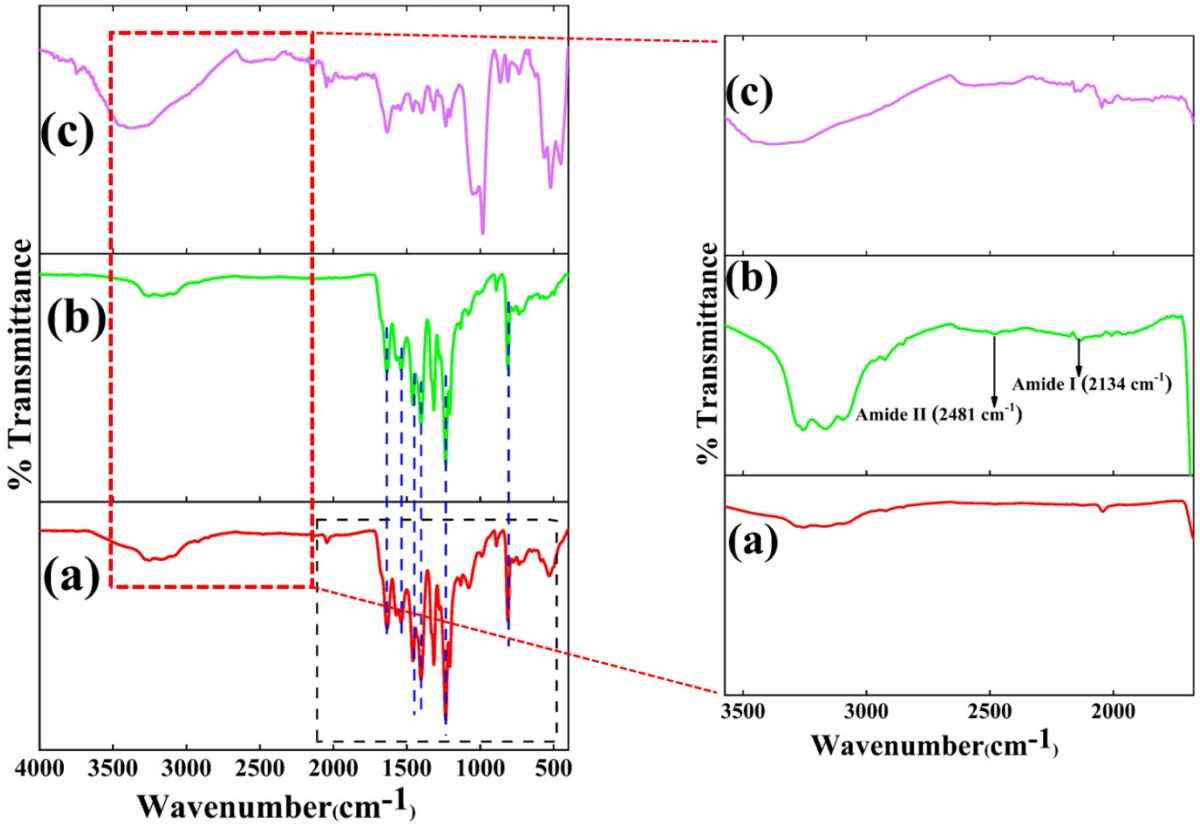

Figure S1: FT-IR spectra of different electrodes modified with anti-IL8 antibodies and BSA molecules (a) APTES@g-C_3N_4/ITO; (b) anti-IL8/APTES@g-C_3N_4/ITO; and (c) BSA/anti-IL8/APTES@g-C_3N_4/ITO.

S3: Optimization studies

(i) Exploration of Optimal Parameters for Electrophoretic Deposition: Voltage and Duration

The electrophoretic deposition (EPD) process involved coating an ITO substrate with a thin film of the APTES@g-C_3N_4. To prepare the solution, APTES@g-C_3N_4 (1 mg mL^{-1}) was ultrasonicated in DI water for 3 hours. Hydrolyzed ITO was selected as the substrate for the deposition. The EPD was conducted using a two-electrode system. The colloidal solution, containing 4 mL acetonitrile, 200 µL APTES@g-C_3N_4 solution (1 mg mL^{-1}), and 20 µL

magnesium nitrate solution (0.5 M) as a supporting electrolyte, was employed in the cell. The ITO served as the cathode (working electrode), while a platinum wire acted as the anode. These electrodes were positioned opposite each other with a 1 cm gap. A uniform film of the APTES@g-C_3N_4 on the ITO substrate was achieved under optimized conditions, specifically at 60 V for duration of 120 seconds. Details of the optimization study can be found in Table S1.

Table S1: Optimization study of Voltage and time during electrophoretic deposition (EPD)

S. No.	Voltage (V)	Time (min)	Result
1	30	1	No deposition on ITO
2	40	2	No deposition on ITO
3	50	1:30	Non uniform deposition on ITO
4	**60**	**1:30**	**uniform deposition on ITO**
5	**60**	**2**	**uniform deposition on ITO**
6	70	1:30	Burning of ITO
7	80	1	Burning of ITO

ii) The concentration of anti-Sp17

The concentration of anti-IL8 plays a crucial role in the successful fabrication of a high-performance biosensor by influencing its detection capability. In order to maximize the signal, the quantity of anti-IL8 was fine-tuned. Six different concentrations of anti-IL8 ranging from 10 to 70 μg mL^{-1} were experimented with, and the outcomes are presented in Fig. S4(c). The DPV method was employed to identify the optimum concentration of anti-IL8 bound to the APTES@g-C_3N_4/ITO electrode. The electrode's peak current exhibited an increasing trend with rising anti-IL8 concentration within the range of 10-50 μg mL^{-1}, reaching saturation at 50 μg mL^{-1}, beyond which no further increase in current intensity was observed (Fig. S4(c)). Lower concentrations of anti-IL8 (10-40 μg mL^{-1}) resulted in a diminished signal, and signals remained constant with increased concentrations of anti-IL8 (50-70 μg mL^{-1}). Consequently, a dilution of 50 μg mL^{-1} of anti-IL8 was selected for subsequent immobilization onto the APTES@g-C_3N_4/ITO electrode.

iii) Incubation time for anti-Sp17 antibodies

An essential optimization factor is the incubation time of the bio-recognition element. This parameter holds significance as it directly impacts the binding efficiency of the antibody. The APTES@g-C_3N_4-modified ITO electrode was subjected to incubation in a PBS solution

containing antibodies with EDC: NHS for varying durations (1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h and 8 h). Incubation times ranging from 1 to 5 hours proved insufficient for antibody binding to the ITO electrode surface. Notably, the signals at 6 h to 8 h were nearly identical, displaying the highest current (Fig. S4(d)). Consequently, a 6-hour incubation time was selected as the optimal duration for binding the bio-recognition molecule to the sensing interface, chosen for its efficiency and time-saving attributes.

iv) Concentration of BSA

The alterations in DPV peak current for the BSA/anti-IL8/APTES@g-C_3N_4/ITO immunoelectrode in response to varied BSA concentrations (ranging from 0.1 % to 4 %) are depicted in Fig. S4(e). An upward trend in peak current was noticed within the BSA concentrations of 0.1% to 2%, reaching saturation thereafter. Consequently, 2% emerged as the chosen and optimal BSA concentration for subsequent experiments.

v) The effect of the redox couple i.e. {[Fe (CN)$_6$] $^{3-/4-}$}

To observe the effect of the redox couple {$[Fe(CN)_6]^{3-/4-}$} on both electrodes i.e., APTES@g-C_3N_4/ITO and BSA/anti-IL8/APTES@g-C_3N_4/ITO immunoelectrode, CV technique was carried out in phosphate buffer saline (PBS) solution and then in PBS (0.2 M) containing 5 mM {$[Fe(CN)_6]^{3-/4-}$} as redox coupler, as shown in Fig. S4 (a) and (b). Fig. S4 indicates that PBS did not produce any strong oxidation or reduction peaks, whereas the addition of the redox couple resulted in prominent redox peaks for both electrodes, demonstrating that it increased the charge carrier/electron transfer. Therefore, PBS with 5 mM {$[Fe(CN)_6]^{3-/4-}$} as redox couple mediator was chosen as the optimal redox buffer for all the electrochemical experiments.

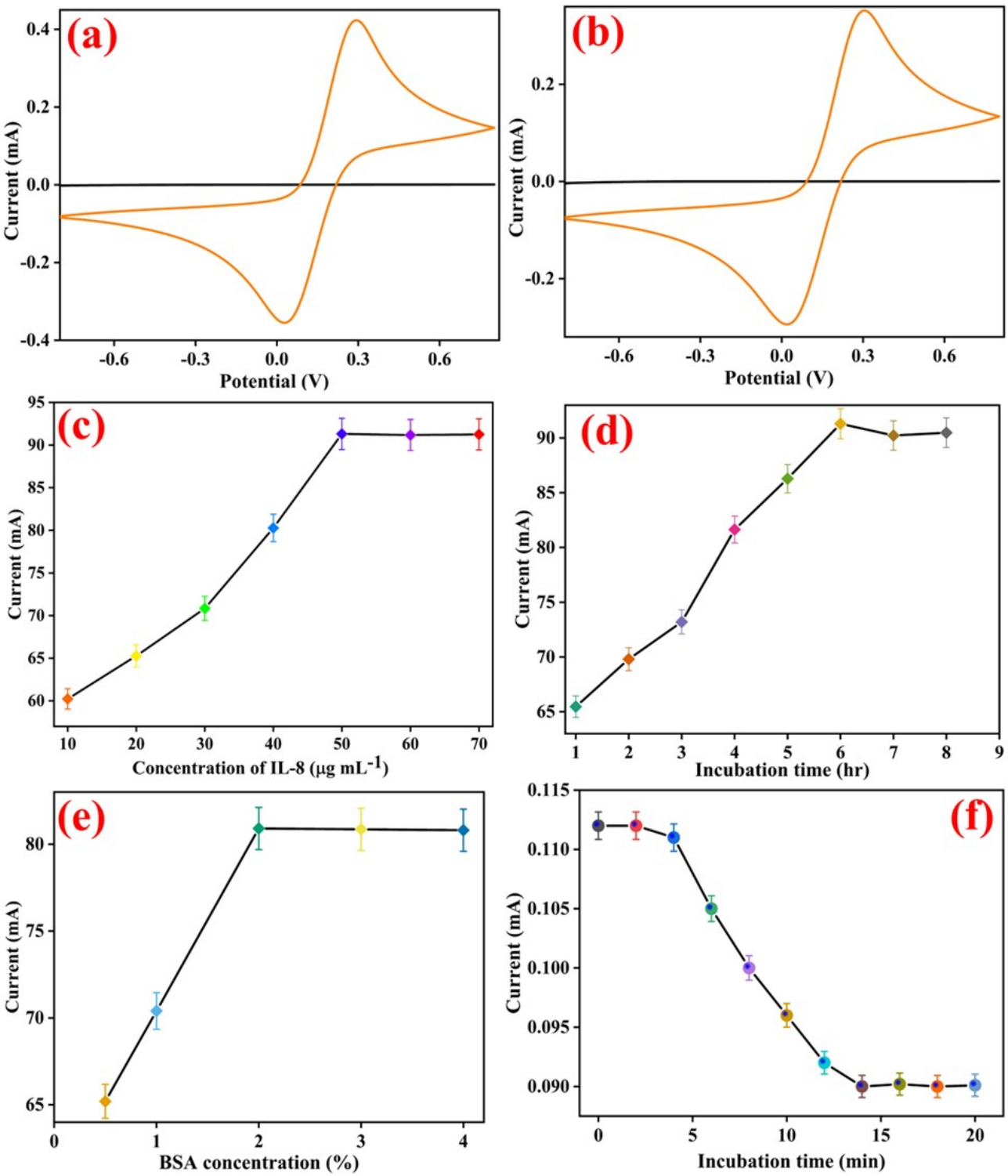

Figure S2: Optimization studies. Effect of redox species $\{[Fe(CN)_6]^{3-/4-}\}$ on (a) APTES@g-C_3N_4/ITO electrode, and (b) BSA/anti-IL8/APTES@g-C_3N_4/ITO immunoelectrode; effect of different concentrations of anti-IL8 (c); effect of different incubation time (e); effect of BSA concentration; and (f) response time study. The error bars are obtained via three independent experiments.

Table S2: Optimized Parameters for Experimental Conditions

Variable	Tested	Selected
EPD parameter of APTES@g-C_3N_4 deposition (voltage & time)	Voltage- 30, 40, 50, 60, & 70 Time- 60, 90 & 120 s	60 V and 120s
anti-IL8 concentration	10, 20, 30, 40, 50, 60, & 70 μg mL^{-1}	50 μg mL^{-1}
anti-IL8 incubation time	1, 2, 3, 4, 5, 6, 7 h, & 8 h	6 h
BSA concentration	0.1, 0.2, 0.5, 1, 1.5, & 2 %	2 %
pH	6.0, 6.5, 7.0, 7.5, & 8.0	7.0
Response time	2, 4, 6, 8, 10, 15, 20 & 25 min	15 min

S4: Kinetic interface studies

The kinetic interface properties of the BSA/anti-IL8/APTES/g-C_3N_4/ITO and APTES@g-C_3N_4/ITO electrodes were evaluated to investigate electron dynamics. Important parameters included the diffusion constant (D), surface concentration of redox species (γ*), effective surface area (Ae), and standard heterogeneous electron transfer rate constant (Ks).

(i) Diffusion co-efficient (D): -

At the interface between the immunoelectrode surface and the electrolyte, which included the redox couple $[Fe(CN)_6]^{3-/4-}$, the diffusion co-efficient (D) was estimated using the Randles-Sevcik equation [8]:

$$Ip = (2.69\times10^5)\, C\, n^{3/2}\, D^{1/2}\, \upsilon^{1/2}\, A \quad \text{Eq. (S1)}$$

Here, the peak current of the electrodes is represented by Ip (Ipa or Ipc), the number of electrons involved in the redox event is indicated by 'n' (in this case, 1), A is the active area of surface of immunoelectrode (0.25 cm^2), D is the diffusion coefficient (cm^2s^{-1}), C is the electrolyte species concentration (5 mM), and υ is the scan rate (50 mVs^{-1}).

In comparison to the APTES@g-C_3N_4/ITO electrode (31.95×10^{-12} cm^2 s^{-1}), the lower value of D observed for the BSA/anti-IL8/APTES@g-C_3N_4/ITO bioelectrode (19.73×10^{-12} cm^2s^{-1}) suggests a faster electron transfer kinetics at the electrolyte/electrode interface, leading to an improved analytical efficiency of this biosensor. Therefore, the working electrode's surface area and the electro-active species concentration affect the diffusion coefficient.

(ii) Effective electroactive surface area (Ae): -

To ascertain the effective and efficient electroactive surface area (Ae) of the unmodified electrode APTES@g-C_3N_4/ITO and the bioelectrode modified with anti-IL8 and BSA (BSA/anti-IL8/APTES@g-C_3N_4/ITO), the computed value of D was integrated into the subsequent equation:

$$A_e = \frac{S}{(2.69\times10^5)n^3\,CD^{1/2}} \quad \text{..... Eq. (S2)}$$

It is found that the BSA/anti-IL8/APTES@g-C_3N_4/ITO bioelectrode has an electroactive area (Ae) of 0.670 mm^2. This result suggests that, in comparison to the APTES@g-C_3N_4/ITO electrode (0.329 mm^2), it is more appropriate, with more reaction sites per unit volume, due to the immobilization of biomolecules (i.e., BSA and antibody).

(iii) Electroactive ionic species (γ*): -

Furthermore, the Brown-Anson equation, which is given as follows, was used to calculate the surface concentration of the absorbed electroactive ionic species (denoted as γ* in mol cm^2) for the corresponding electrodes:

$$I_p = \frac{n^2F^2\gamma^*\,A\,\upsilon}{4\,R\,T} \quad \text{..... Eq. (S3)}$$

Here, the peak current is denoted by Ip (Ipa or Ipc), the electrode surface area is represented by A, the surface concentration of the absorbed electro-active species is represented by γ, the scan rate is denoted by υ (Vs^{-1}), the ambient temperature (298 K) is represented by T, the Faraday constant (96485 C mol^{-1}), and the gas constant (8.314 mol^{-1} K^{-1}) is represented by R. The surface concentration (γ*) of the BSA/anti-IL8/APTES@g-C_3N_4/ITO bioelectrode is approximately 2.86×10^{-8} mol cm^2, which is lower than that of the APTES@g-C_3N_4/ITO electrode (3.64×10^{-8} mol cm^2). This discrepancy suggests that the presence of g-C_3N_4 surface

has improved its electrocatalytic performance, making it appropriate for biosensing applications.

(iv) Heterogeneous electron transfer rate constant (Ks): -

The reversibility of electron transfer kinetics is dependent on the heterogeneous electron transfer rate constant (Ks) as well as the scan rate. The Laviron Equation (11) shows that the values of Ks for the APTES@g-C_3N_4/ITO electrode and the BSA/anti-IL8/APTES@g-C_3N_4/ITO bioelectrode are 0.299 s^{-1} and 0.197 s^{-1}, respectively. Higher Ks values for the APTES@g-C_3N_4/ITO electrode indicate a quicker electron exchange between the redox species in the electrolyte and the electrode surface.

$$K_s = \frac{mn\,Fv}{RT} \quad \text{..... Eq. (S4)}$$

where m is the peak-to-peak separation of potentials (V)

S5: ***Calculation of number of molecules in IL8 at LOD of 0.04 ng mL^{-1}:***

Molecular weight of IL8 = 8 kDa or 8×10^3 g mol^{-1}

Concentration (strength) of IL8 solution at LOD = 0.04 ng mL^{-1} or 4×10^{-8} g L^{-1}

Thus, molarity of solution = strength/ molecular weight

$= 4\times10^{-8}$ g L^{-1}/ 8×10^3 g mol^{-1}

$= 5\times 10^{-12}$ mol L^{-1}

Number of molecules of IL8 in this concentration

= Molarity × Avogadro number

$= 5\times 10^{-12}$ mol $L^{-1}\times 6.022 \times 10^{23}$ molecules mol^{-1}

$= 3.0 \times 10^{10}$ molecules L^{-1}

So, number of molecules in 1 L or 10^6 µL of solution = 3.0×10^{10} molecules

Therefore, Number of molecules in 20 µL of solution

$= 3.0 \times 10^{10}$ molecules × 20 µL/10^6 µL

$= 6.0 \times 10^{-3}$ molecules/20 µL

Hence, 20 µL of 0.04 ng mL^{-1} solution contains ~ 6.0×10^{-3} molecules of IL8

S6: Regeneration and Shelf-life studies

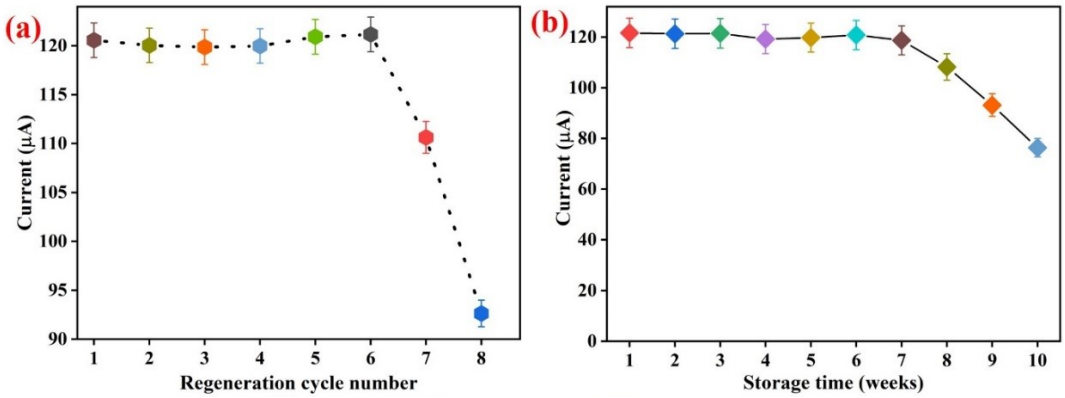

Figure S3: (a) The impact of regeneration response on the BSA/anti-IL8/APTES@g-C_3N_4/ITO immunoelectrode; (b) Stability analysis of the developed BSA/anti-IL8/APTES@g-C_3N_4/ITO immunoelectrode.