Supplementary Information

Disposable Paper-Based Electrochemical Biosensor Employing g-C_3N_4/Carbon Dots and Toll-like Receptor for Ultrasensitive Detection of Gram-Negative Bacteria

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1. Materials and Reagents

For this study, Graphite powder (20 µm), Ethyl cellulose (EC), n-Hexanol, Triton-X, 25 % Glutaraldehyde solution, Citric acid, and Dialysed bags were purchased from Sigma Aldrich. From Sisco Research Laboratories Pvt. Ltd, India, Melamine powder, Solid Paraffin wax, Bleaching solution (NaOCl), Potassium chloride (KCl), Ammonia solution (NH_4OH) and Sulfuric acid (H_2SO_4) were procured. All chemicals were used as received without further purification. Ivory drawing paper was ordered from Amazon, India and the Conductive silver ink was obtained from Thermo-scientific. The TLR-4/MD-2 bioreceptor were purchased from Sales Nulife (Abcam). All the experiments including cleaning and solution preparation were carried out using DI water with a resistivity of 18.2 $M\Omega/cm^{-1}$.

2. TEM Analysis

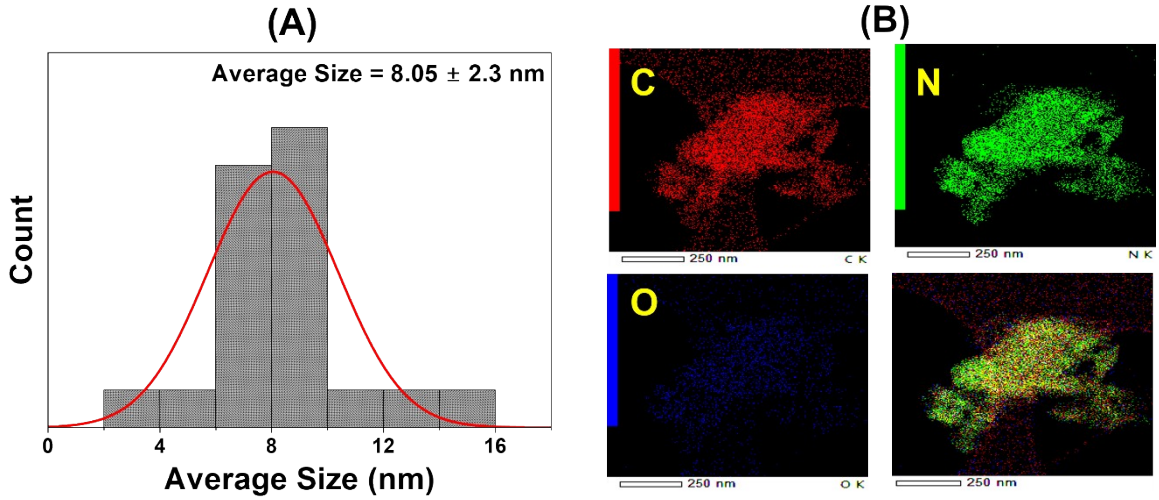


Fig. S1(A) Average particle size of the carbon dots in the composite material, and **(B)** Elemental mapping of g-C_3N_4/CDs composite.

3. Bioelectrode Modification

3.1. FTIR and XPS analysis during modification steps

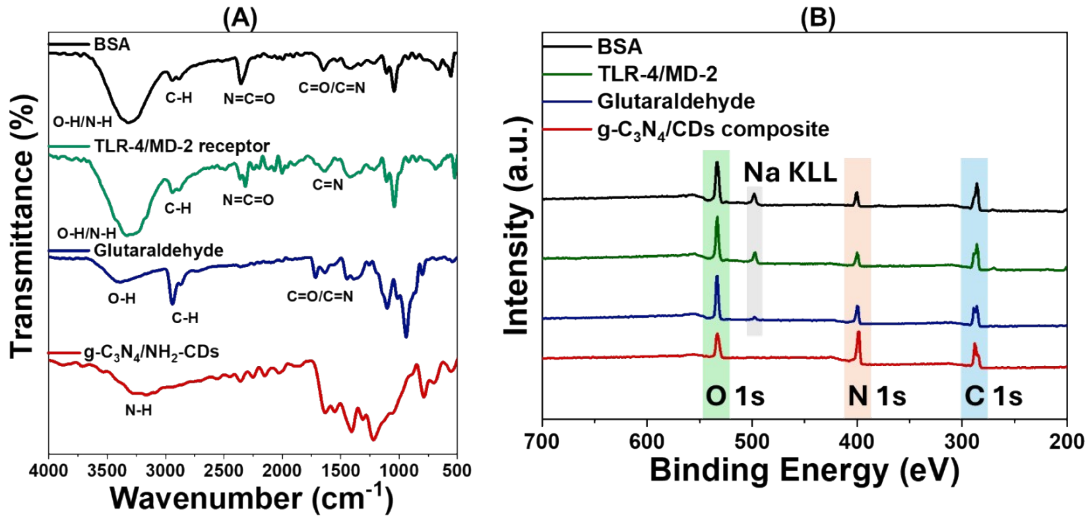


Fig. S2(A)FTIR analysis, and **(B)** XPS analysis of the layer-by-layer bioelectrode modification.

Table S1. Surface elemental composition of the bioelectrode obtained from XPS.

Modification	C (%)	N (%)	O (%)
g-C_3N_4/CDs	48.8	34	17.2
Glutaraldehyde	53.9	18.8	27.3
TLR-4/MD-2	59.1	13.2	27.7
BSA	57.7	12.2	30

3.2. SEM and AFM analysis during modification steps of bioelectrodes

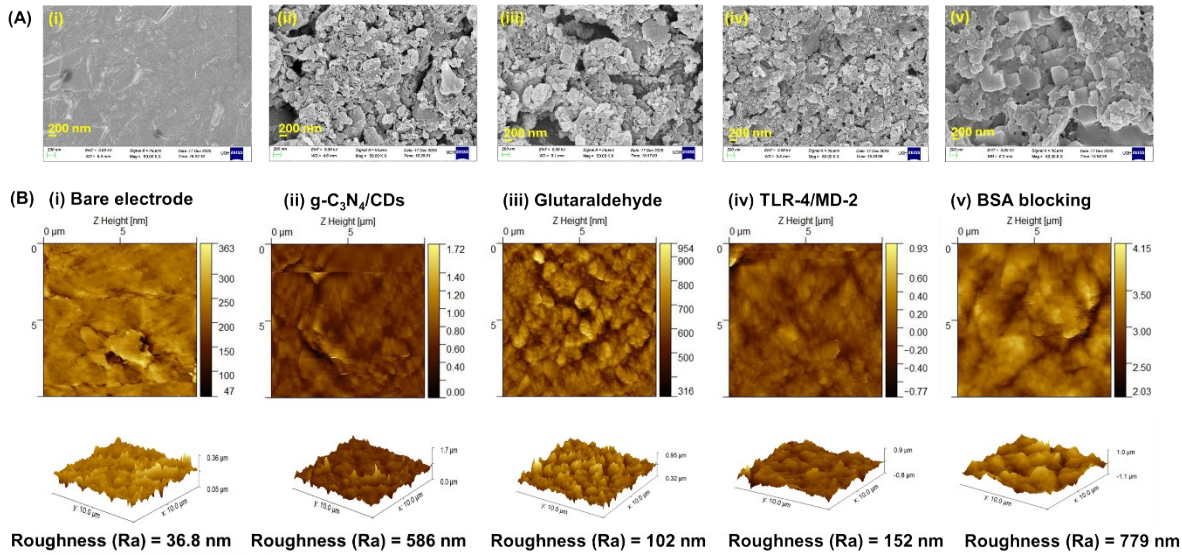


Fig. S3. (A) SEM morphology study, **(B)** AFM analysis of the layer-by-layer modification step of the bioelectrodes.

3.3. Electrochemical characterisation

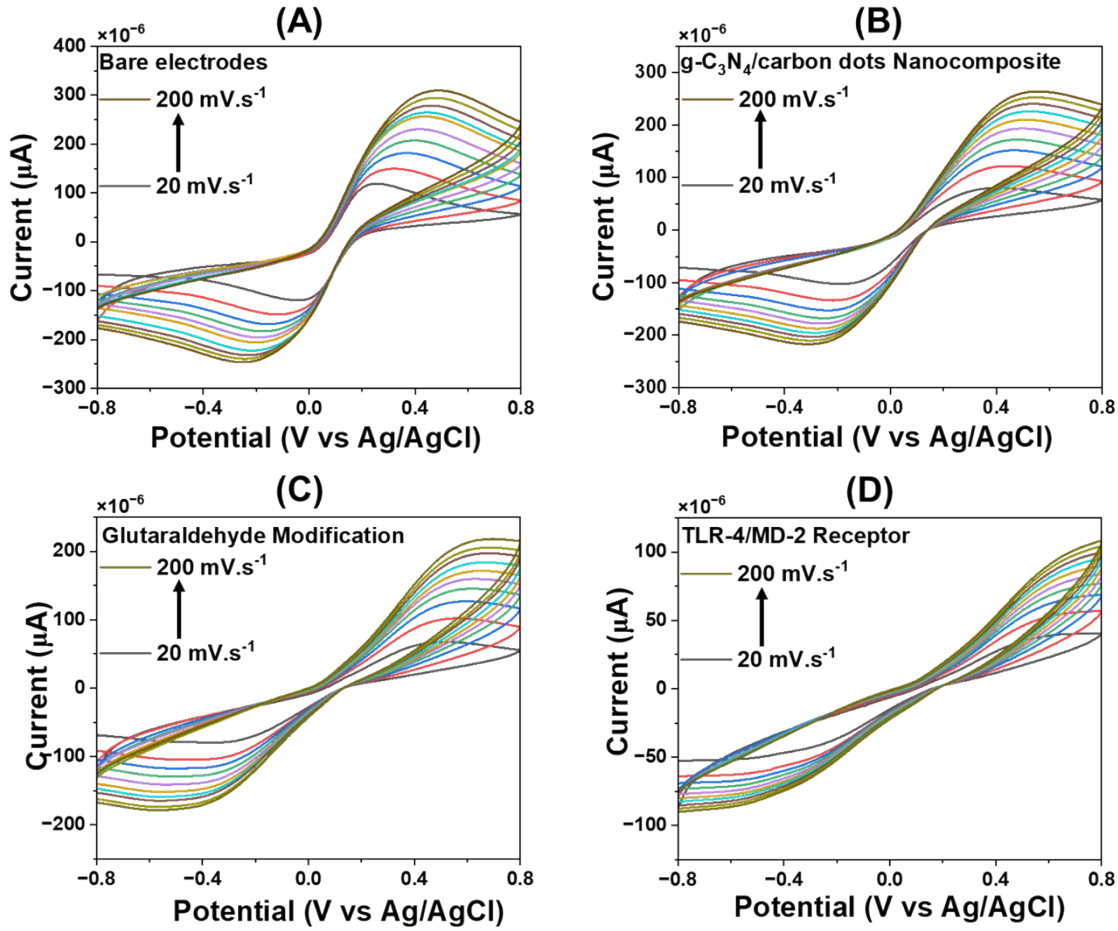


Fig. S4. (A) The Cyclic voltammetry (CV) study at varying scan rates from 20 mV/s to 200 mV/s of bare paper electrodes, **(B)** modified with g-C_3N_4/CDs composite, **(C)** activation via glutaraldehyde bio-linker, and **(D)** after immobilisation of the TLR-4/MD-2 bio-receptor.

4. Determination of Electrochemically active surface area

The electrochemically active area of the bioelectrodes was obtained with the slope of the intensity of the anodic peak current and the square root of the scan rate from the Randle-Sevcik equation (**Equation S1**) for the electron-electron transfer process.

$$\mathbf{I_{pa} = (2.69 \times 10^5)\, n^{3/2}\, D^{1/2}\, C_o\, A_e\, \nu^{1/2}} \quad \text{... (Equation S1)}$$

Where I_{pa} = anodic current response

n = Number of electrons involved in the redox process (n=1)

D = Diffusion coefficient of the electroactive species = 6.7×10^{-6} cm^2/s

C_o = Concentration of the electroactive species = 10 mM = 10^{-5} mol/cm^3

A_e = Electrochemically active area

ν = Scan rate.

So, equation S1 can be modified as

$$\mathbf{Slope\ (A/V^{1/2}) = (2.69 \times 10^5)\, n^{3/2}\, D^{1/2}\, C_o\, A_e}$$

$$\mathbf{Electrochemical\ Active\ (A_e) = Slope/(2.69 \times 10^5)\, n^{3/2}\, D^{1/2}\, C_o}$$

The slope of the anodic sweep from the calibration curve of the bare electrode was obtained as 19.9 $\mu A/(mV/s)^{1/2}$.

Slope = 19.9 $\mu A/(mV/s)^{1/2}$ = 19.9 x 10^{-6} A /$(10^{-3}$ V/s$)^{1/2}$ = 6.29 x 10^{-4} $A/(V/s)^{1/2}$

Thus, the electrochemical active area (A_e) = Slope/(2.69 x 10^5) $n^{3/2}$ $D^{1/2}$ C_o = 6.29 x 10^{-4} $A/(V/s)^{1/2}$/(2.69 x 10^5) $n^{3/2}$ $D^{1/2}$ C_o **= 0.09 cm^2.**

Table S2: Electrochemical active area calculation using the Randles–Sevcik equation.

Electrochemically active surface area (ECAA) calculation

Modification Step	Slope ($\mu A/(mV/s)^{1/2}$)	Slope ($A/V/s^{1/2}$)	ECAA (cm^2)
Bare DPE	**19.9**	**6.29 x 10^{-4}**	**0.09**
g-C_3N_4/NH_2-Carbon dots Nanocomposite	**19.1**	**6.04 x 10^{-4}**	**0.086**
Glutaraldehyde biolinker	**14.9**	**4.71 x 10^{-4}**	**0.067**
TLR-4/MD-2 bioreceptor Immobilisation	**5.6**	**1.77 x 10^{-4}**	**0.025**

5. Determination of Surface Coverage

The surface coverage of the modified bioelectrode was indirectly calculated using the Brown-Anson model, **$Ip = n^2F^2\Gamma A_e\nu/4RT$ … (Equation S2)**

$\Gamma = n^2F^2A_e\nu/4RTIp$

where:

Ip = peak current (A)

n = number of electrons transferred (n=1)

F = Faraday constant (F= 96485 C mol^{-1})

A = electrode active area (cm^2)

Γ = surface coverage (mol cm^{-2})

ν = scan rate (V s^{-1}) = **0.40 V/s**

R = gas constant (8.314 J mol^{-1} K^{-1})

T = absolute temperature (≈ 298 K if room temp)

Table S3: Calculation of surface coverage using the Brown-Anson equation.

Calculation of Surface Coverage at a scan rate of 0.40 V/s

Modification Step	Ipa (µA)	Ae (cm^2)	Γ (nmol/cm^2)
g-C_3N_4/NH_2-Carbon dots Nanocomposite	**122**	**0.086**	**37.8**
Glutaraldehyde biolinker	**101**	**0.067**	**40.1**
TLR-4/MD-2 bioreceptor Immobilisation	**66.83**	**0.025**	**71.0**

6. Selectivity and Interference Study

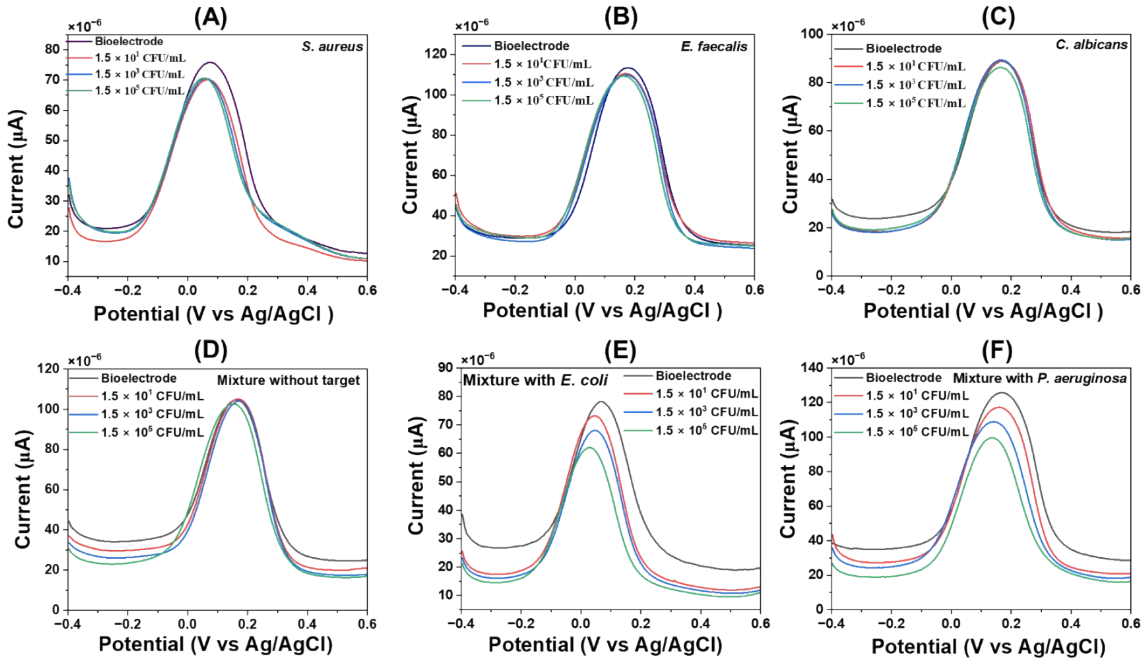
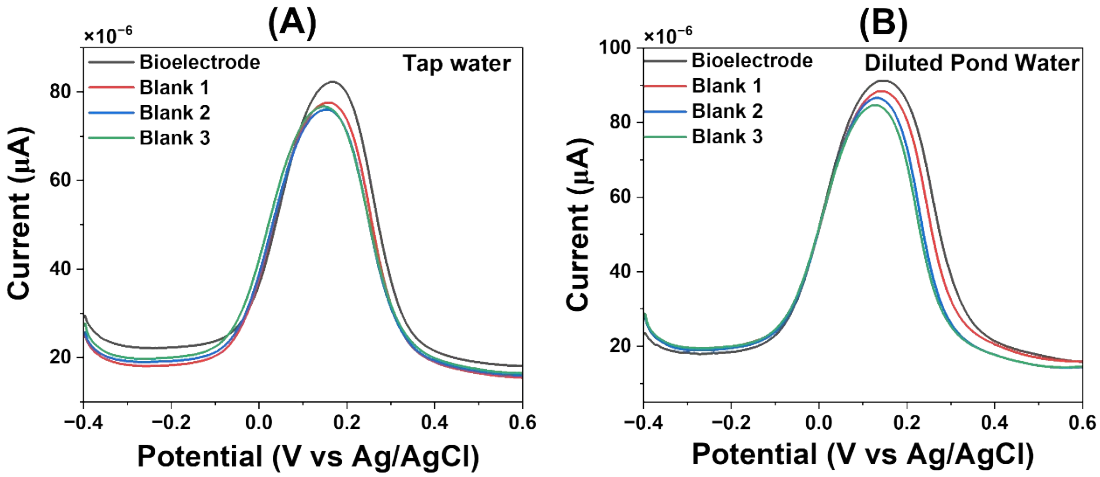


Fig. S5. Selectivity study of the bioelectrodes with **(A)** *S. aureus*, **(B)** *E. faecalis*, **(C)** *C. albicans*, **(D)** Interference study of the bioelectrodes with Mixture without gram-negative species, **(E)** Mixture of bacteria spiked with *E. coli*, and **(F)** Mixture of bacteria spiked with *P. aeruginosa*.



7. Real sample Blank Study

Fig. S6. (A) The blank current response of the tap water and **(B)** diluted pond water.