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Graphene oxide/Cu–MOF-based electrochemical immunosensor for the simultaneous detection of *Mycoplasma pneumoniae* and *Legionella pneumophila* antigens in water

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The combination of copper–metal organic framework (Cu–MOF) with graphene oxide (GO) has received growing interest in electrocatalysis, energy storage and sensing applications. However, its potential as an electrochemical biosensing platform remains largely unexplored. In this study, we introduce the synthesis of GO/Cu–MOF nanocomposite and its application in the simultaneous detection of two biomarkers associated with lower respiratory infections, marking the first instance of its use in this capacity. The physicochemical properties and structural elucidation of this composite were studied with the support of XRD, FTIR, SEM and electrochemical techniques. The immunosensor was fabricated by drop casting the nanocomposite on dual screen-printed electrodes followed by functionalization with pyrene linker. The covalent immobilization of the monoclonal antibodies of the bacterial antigens of *Mycoplasma pneumoniae* (*M. pneumoniae*; *M. p.*) and *Legionella pneumophila* (*L. pneumophila*; *L. p.*) was achieved using EDC-NHS chemistry. The differential pulse voltammetry (DPV) signals of the developed immunosensor platform demonstrated a robust correlation across a broad concentration range from 1 pg/mL to 100 ng/mL. The immunosensor platform has shown high degree of selectivity against antigens for various respiratory pathogens. Moreover, the dual immunosensor was successfully applied for the detection of *M. pneumoniae* and *L. pneumophila* antigens in spiked water samples showing excellent recovery percentages. We attribute the high sensitivity of the immunosensor to the enhanced electrocatalytic characteristics, stability and conductivity of the GO–MOF composite as well as the synergistic interactions between the GO and MOF. This immunosensor offers a swift analytical response, simplicity in fabrication and instrumentation, rendering it an appealing platform for the on-field monitoring of pathogens in environmental samples.

Keywords Respiratory infections, Simultaneous detection, Electrochemical biosensor, Bacterial pathogens

Respiratory infections (RIs) present a significant global health challenge, marked by substantial morbidity and mortality^{1,2}. They have been recognized as the fourth-leading global cause of mortality by the World Health Organization (WHO). RIs caused nearly three million deaths in 2016, translating to 40 deaths per 100,000³. Lower respiratory infections (LRIs), such as pneumonia and bronchiolitis, notably contribute to hospital admissions and in-hospital fatalities, particularly among young children^{4,5}. The recent novel coronavirus (COVID-19) outbreak, triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in late 2019, has intensified the scrutiny on respiratory tract infections as a crucial public health concern⁶.

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The presence of microbial pathogens in water systems poses significant public health risks⁷. Among these pathogens, *Mycoplasma pneumoniae* (*M. pneumoniae*) and *Legionella pneumophila* (*L. pneumophila*) are of particular concern due to their ability to cause severe respiratory infections. *M. pneumoniae* is a common cause of atypical pneumonia, especially in children and young adults, while *L. pneumophila* is responsible for Legionnaires' disease, a severe form of pneumonia that can be fatal if not promptly treated⁸. *M. pneumoniae* is estimated to cause 20–40% of community-acquired pneumonia (CAP) cases in certain populations, whereas *L. pneumophila* accounts for 2–15% of CAP cases globally. The mortality rate for *L. pneumophila* in hospitalized patients is about 10–15%, while for *M. pneumoniae*, it is typically under 1%, primarily impacting those with severe underlying conditions^{9,10}. Both pathogens can thrive in water environments, making water systems potential reservoirs for infection. *L. pneumophila*, in particular, is known to proliferate in warm water systems such as cooling towers, hot tubs, and plumbing systems. Human infections primarily occur through inhaling contaminated aerosols^{11–13}. Therefore, the detection of these antigens in water is crucial for preventing outbreaks and ensuring public safety.

Cultures from throat swab or sputum specimens may reveal the presence of *M. pneumoniae* and *L. pneumophila*, although discernible growth is rare before 1 week of incubation⁹. In recent years, various analytical techniques for quantitatively determining bacterial pathogens, including Enzyme-linked immunosorbent assays (ELISA)¹⁴, accelerated strand exchange amplification (ASEA)¹⁵, immunochromatographic strips^{16,17} and polymerase chain reaction (PCR) analysis^{18–20}, have undergone exploration. While these methods exhibit sensitivity, their practical utility is constrained by the necessity for proficient operators, intricate sample pre-treatments, costly instrumentation, and time-consuming procedures, impeding their widespread application in bacterial pathogen detection. Electrochemical immunosensors have been widely developed for pathogens detection, utilizing different materials^{21–26}. These methods offer cost saving potential, ease of use and high scalability. Park et al. documented the use of zinc oxide nanorods on a Ti/Au electrode for highly sensitive *L. pneumophila* detection with specific antibodies²⁷. However, this immunosensor utilized an enzymatic assay which require multiple washing steps and reporter reagents limiting their practical applicability²⁷. Additionally, Li et al. reported a dual electrochemical and fluorescent immunosensor platform designed for the specific detection of *L. pneumophila* by covalently immobilizing fluorophore-conjugated antibodies on Au chips²⁸.

Metal–organic frameworks (MOFs) are crystalline materials, merging organic linkers and metal ions to form frameworks with high surface areas, customizable pores, catalytic sites, and functional groups^{29,30}. Their advantageous features, such as the remarkable surface area and tuneable porosity, make MOFs versatile for applications in catalysis, gas adsorption/separation, supercapacitors, chemical sensors, fuel cells, and batteries³¹. Current efforts focus on integrating transition metal-based MOFs into electrochemical sensing platforms^{32,33}. Transition metals (Cu, Zn, Mn, Co and Ni) within the framework serve as active sites, expediting electrocatalytic reactions, while organic ligands act as adsorption sites for specific molecules³⁴. Recent studies underscore the use of various MOF materials as electrochemical sensor platforms for the detection of vital diagnostic biomarkers. For example, Li et al. employed AuNPs with ZIF-8 as a sensor substrate for electrochemical immunosensing of alpha-fetoprotein³⁵ and Dai et al. utilized a cobalt-based MOF for detecting prostate-specific antigen³⁶. Additionally, Bajpai et al. reported the use of a zirconium-based MOF for detecting the hepatitis B virus surface antigen³⁷, while Ravipati et al. showcased a nickel-based MOF for the sensitive detection of immunoglobulin G³⁸. Despite copper(II) benzene-1,3,5-tricarboxylate (Cu-BTC) traditionally finding use in gas storage, catalysis, and energy storage applications, its potential in electrochemical sensors remains a scientific focus^{33,39}. Electrochemical sensors based on the Cu-BTC electrocatalytic activity was used to detect substances like malachite green⁴⁰, tartrazine⁴¹, dopamine⁴², paracetamol⁴³, digoxin⁴⁴, and 2,4-dichlorophenol (DCP)⁴⁵. However, despite their numerous advantages, MOFs encounter significant limitations, primarily in their low electrical conductivity^{46,47} and, in some cases, susceptibility to instability in humid environments⁴⁸. To address this issue, the development of MOFs/Graphene or Graphene oxide (GO) composites has garnered attention. These composites have shown promising characteristics, especially in electrocatalysis, energy storage and gas sensing applications, owing to the synergistic effects derived from combining the two materials^{49–57}. For instance, Wang et al. reported the fragmentation of GO to actively participate in forming Cu–MOF with HKUST-1 linker, resulting in increased redox activity, surface area, and electrical conductivity⁵⁸. Patit et al. demonstrated a synergistic effect between GO and MOF, enhancing porosity and adsorption⁵⁹. The MOF-rGO combination not only facilitates active material utilization but also synergistically improves mechanical strength, conductivity and biocompatibility^{60–62}. Nguyen et al. also demonstrated the synthesis of GO/Cu–MOF and its utilization as a sensor substrate for 2,4-DCP detection⁴⁵. Allahbakhsh et al. reported that the composite of GO/Cu–MOF has better anti-bacterial activity against *Escherichia coli* and *Staphylococcus aureus* than the virgin MOF⁶¹. However, the use of Cu–MOF/GO as an electrochemical biosensing platform, including bioreceptors, remains largely unexplored.

Building upon the preceding discussions, we synthesized GO/Cu–MOF composite using green ultra-sonication-assisted stirring method. The composite was then used to fabricate a label-free electrochemical immunosensor for the simultaneous detection of *M. pneumoniae* and *L. pneumophila* antigens. The biosensor functions by impeding the mass diffusion of the known redox probe $[\text{Fe}(\text{CN})_6]^{3-/4-}$ to the electrode surface through specific antibody–antigen binding. Due to its large surface area, high porosity and synergetic effect between GO and MOF, GO/Cu–MOF modified SPE electrode offers high-density immobilizing sites and facilitates improved interfacial charge transfer via the GO backbone. The combined strengths of this hybrid GO/Cu–MOF yield a biocompatible surface with enhanced electrochemical properties. Analytical methods were fine-tuned to enhance performance, encompassing linear determination range, sensitivity, selectivity, reproducibility, and successful application in environmental samples.

Experimental section

Materials

Copper nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$; 98%), 1,3,5-benzenetricarboxylic acid (BTC), phosphate buffer saline (PBS) pellets, 1-pyrenebutyric acid (PY), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-Hydroxy succinimide (NHS), *N,N*-dimethylformamide (DMF), ethanol, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), potassium ferricyanide ($\text{K}_3[\text{Fe}(\text{CN})_6]$), potassium ferrocyanide ($\text{K}_4[\text{Fe}(\text{CN})_6]$) and bovine serum albumin (BSA) were obtained from Merck, USA. Graphene oxide (GO) suspension (4 mg/mL) was purchased from Graphenea (USA). Mouse anti-*Mycoplasma pneumoniae* P1 (6537), mouse anti-*Legionella pneumophila* (LN29) antibodies, *Legionella pneumophila*, mycoplasma pneumoniae antigens and interferents such as canine adenovirus 1 (CAV1), human metapneumovirus (hMPV) B, Influenza A (Cambodia) virus (IAV), Influenza B (Austria) virus (IBV), and respiratory syncytial virus B (RSVB) Lysate antigens were purchased from the Native Antigen company, United Kingdom. Deionized water obtained from the Millipore system was utilized for all the experimental studies. Disposable dual carbon screen-printed electrodes (SPEs) were obtained from Metrohm, Switzerland. The dual electrode model (DRP-C1110) features two elliptical carbon working surfaces (6.3 mm² each), a shared carbon counter electrode, and a silver reference electrode. These elements are all screen-printed on a ceramic substrate measuring 3.40 × 1.00 × 0.05 cm in length, width, and height, respectively.

Instruments

Fourier transform Infrared (FTIR) analysis was used to identify unique features in the spectra of the Cu–MOF and GO/Cu–MOF hybrid between 500 and 3700 cm^{−1}, using a Bruker Vertex 80v FT-IR instrument. The D2 Phaser X-ray Diffraction (XRD) Powder Diffractometer, operating at 40 kV and 40 mA with Cu-K α radiation ($\lambda = 1.5406 \text{ \AA}$), was used for XRD analysis. Measurements were carried out over a 2θ range from 5° to 50°. The Raman spectra of the modified electrodes were obtained using the WiTec alpha300R Raman spectrometer. Quanta 250 FEG from the Netherlands was utilized for the analysis of scanning electron microscopy (SEM) and Energy dispersive X-ray (EDAX). The voltammetric electrochemical measurements were conducted using the MultiEmStat4 potentiostat (PalmSens) along with support from the MultiTrace software. A dedicated cable connector for the SPE, connecting the electrode to any potentiostat, was acquired from Metrohm, Switzerland. Differential pulse voltammetric (DPV) measurements were conducted within the potential range of −0.5 V to −0.2, employing a scan rate of 0.05 V/s, an amplitude of 0.002 V, and step potential of 0.004 V.

Synthesis of Cu–MOF

A Cu–MOF was synthesized using a solvothermal approach^{63,64}. To initiate the process, 2.0 mmol of 1,3,5-benzenetricarboxylic acid was dissolved in 30 mL of DMF, while simultaneously, 2.0 mmol of $\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$ was dissolved in another 30 mL of DMF. These solutions were amalgamated and continuously stirred for a duration of 30 min. Following this, the resultant mixture was transferred into a 100 mL Teflon-lined stainless-steel autoclave and subjected to a solvothermal reaction at 140 °C for a duration of 8 h. Upon completion of the reaction, the autoclave was allowed to naturally cool to room temperature (RT). The resulting product underwent multiple washes with distilled water and ethanol, was collected via centrifugation, and ultimately dried at 60 °C overnight.

Preparation of the GO/Cu–MOF composite and coating of the electrodes

The synthesis method for the composite is illustrated in Fig. 1A. A mixture of equal volumes of GO (2 mg/mL) and Cu–MOF (2 mg/mL) was combined with 0.5% Nafion. The role of Nafion in the fabrication process is to act as a binder and stabilizer, providing a uniform dispersion of the nanocomposite on the electrode surface and enhancing its mechanical stability and conductivity^{65–67}. The mixture underwent 30 min of ultrasonication (50 kHz and 80 W power) to produce the composite known as GO/Cu–MOF. Following an initial rinse with deionized (DI) water, the carbon working surface of the designated dual electrode was modified by depositing 2 μL of this suspension using drop casting, further the electrode was allowed to dry at RT for 12 h. Subsequently, all modified electrodes were thoroughly washed with DI water in preparation for further experiments.

Immunosensor construction

As shown in Fig. 1B, the fabrication of the immunosensor for *M. pneumoniae* and *L. pneumophila* involved the activation of the GO/Cu–MOF-modified electrodes followed by immobilization of the antibodies. First, 100 μM concentration of PY in PBS buffer, pH 7.4 was drop-casted onto the dual GO/Cu–MOF electrode surface and incubated at RT for 30 min. Subsequently, 10 μL of EDC-NHS (10 $\mu\text{g/mL}$ in 0.01 M PBS, pH 5.5) solution was drop-casted onto the GO/Cu–MOF/PY electrode surface and incubated at RT for 1 h. After each incubation, the electrodes were gently immersed in PBS solution to remove unbound PY and excess EDC-NHS, respectively. The immobilization of monoclonal antibodies for *M. pneumoniae* and *L. pneumophila* was achieved by drop-casting the antibodies (10 $\mu\text{g/mL}$ in 0.01 M PBS, pH 7.4) onto the activated GO/Cu–MOF/PY surface, allowing them to incubate for 3 h. Following washing with PBS buffer to eliminate excess antibodies, the antibodies-modified electrode underwent treatment with a BSA solution (2 μL , 0.1% w/v) as a blocking agent and was incubated for 30 min to prevent non-specific interactions. After a gentle wash in the PBS buffer solution, the as-developed immunosensor was stored at 4–8 °C for further use.

Immunodetection of *M. pneumoniae* and *L. pneumophila* antigens

Detection of *M. pneumoniae* and *L. pneumophila* relied on monitoring the antigen–antibody immune reaction using DPV (Fig. 1C). Solutions containing desired analyte concentrations of *M. pneumoniae* and *L. pneumophila*

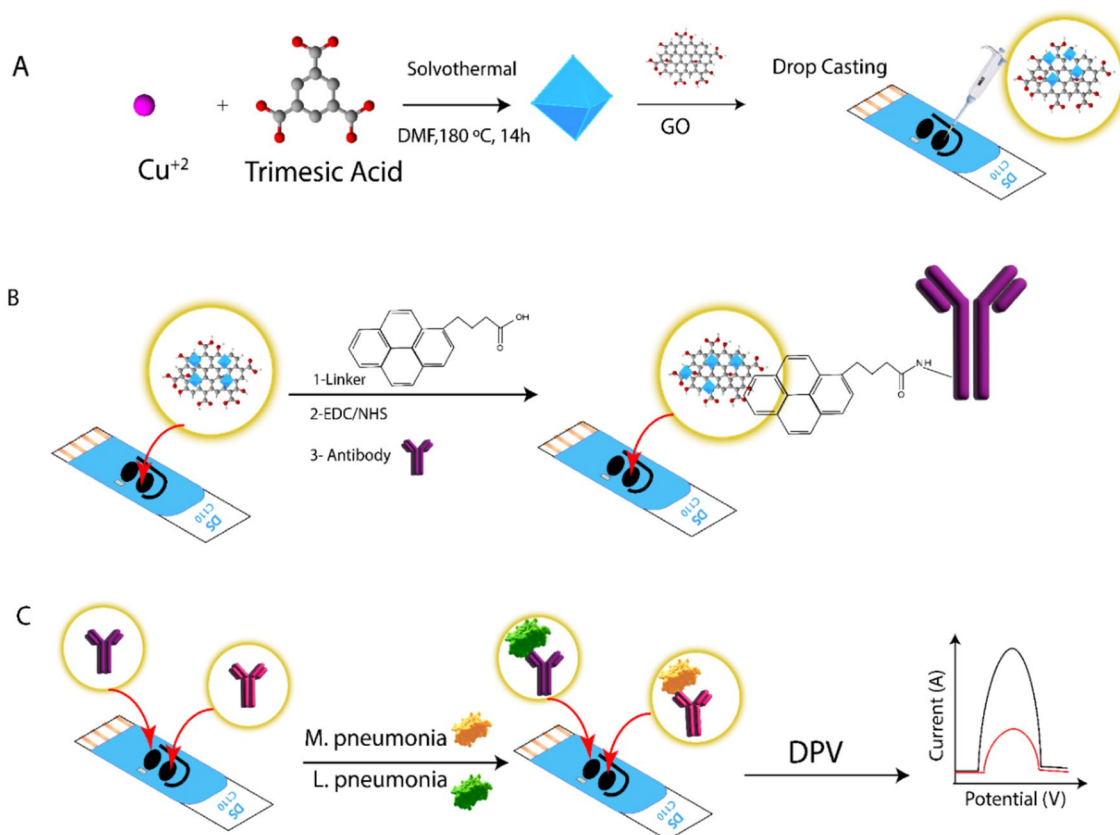


Figure 1. Illustration of (A) the synthesis method used for the preparation of the GO/Cu-MOF composite. (B) The steps for the fabrication of the immunosensor and (C) the dual detection of *M. pneumoniae* and *L. pneumophila* antigens using differential pulse voltammetry.

proteins (1, 10, 50, 100, and 500 pg/mL, and 1, 10, and 100 ng/mL) were prepared in PBS buffer and incubated onto the electrode surface at RT. After 30-min incubation period, the electrodes were washed with PBS buffer and DPV measurements were recorded using the parameters described above. The detection was achieved by observing the change in the reduction peak current response after binding. The typical volume for the measuring solution required to cover the integrated DropSens electrodes was approximately 50 μL for the dual sensor surfaces. All the electrochemical immunosensor measurements were carried out in 0.01 M PBS containing 1 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$, and the experiments were repeated at triplicate.

Results and discussions

Physicochemical characterization of the GO/Cu-MOF composite

To characterize the chemical structures and morphologies of the prepared Cu-MOF and GO/Cu-MOF composite, FTIR, XRD, Raman spectroscopy and SEM analysis were used. FTIR spectra of the Cu-MOF and GO/Cu-MOF composite were analyzed and the spectra was illustrated in Fig. 2a. Synthesized Cu-MOF exhibited various bands at 728, 1114, 1371, 1445, 1542 and 1644 cm^{-1} . In which, 728 and 1114 cm^{-1} corresponds to C-O and Cu-O indicating coordination of the organic linker with copper ions. The organic linker (BTC) displays C-O asymmetric stretching at 1371 and 1542 cm^{-1} characteristic of carboxylate groups forming bonds with metal centers. Bands at 1445 and 1644 cm^{-1} signifies the aromatic C=C stretching of the benzene ring, and C=O stretching of carboxylate groups, respectively⁶⁹. A broad band attributed to O-H stretching is observed spanning 3500–2800 cm^{-1} . This broad band is often indicative of hydrogen-bonded OH groups or moisture within the sample. The FTIR spectrum observed for the Cu-MOF/GO composite indicates that the integration of GO into the MOF did not adversely affect the crystalline structure of Cu-MOF. This is supported by the persistence of characteristic peaks, especially those attributed to the C-O and Cu-O bonds at 1114 and 728 cm^{-1} , which implies that the fundamental chemical structure of the Cu-MOF framework remains intact even after the incorporation of GO. This suggests that the hybrid material retains the essential functional groups and coordination environment of the original MOF, which is crucial for maintaining its desired properties and functionalities.

The compositions of Cu-MOF, GO, and GO/Cu-MOF were examined using XRD (Fig. 2b). In Cu-MOF, distinct peaks were identified at angles of 9.86°, 12°, 13.76°, 17.83°, and 19.38° corresponding to the (220), (222), (400), (500), and (440) planes, respectively. The highly intense peak at 12° (222) confirms the crystalline nature of Cu-MOF^{70,71}. In an inset of Fig. 2b, GO displayed a prominent peak at 10.5°, indicative of the characteristic peak of the 001 crystal plane, revealing the interlayer spacing in the GO structure⁷². In the GO/Cu-MOF composite, the characteristic Cu-MOF peak persisted with a slight shift in the 222 planes, suggesting interaction with

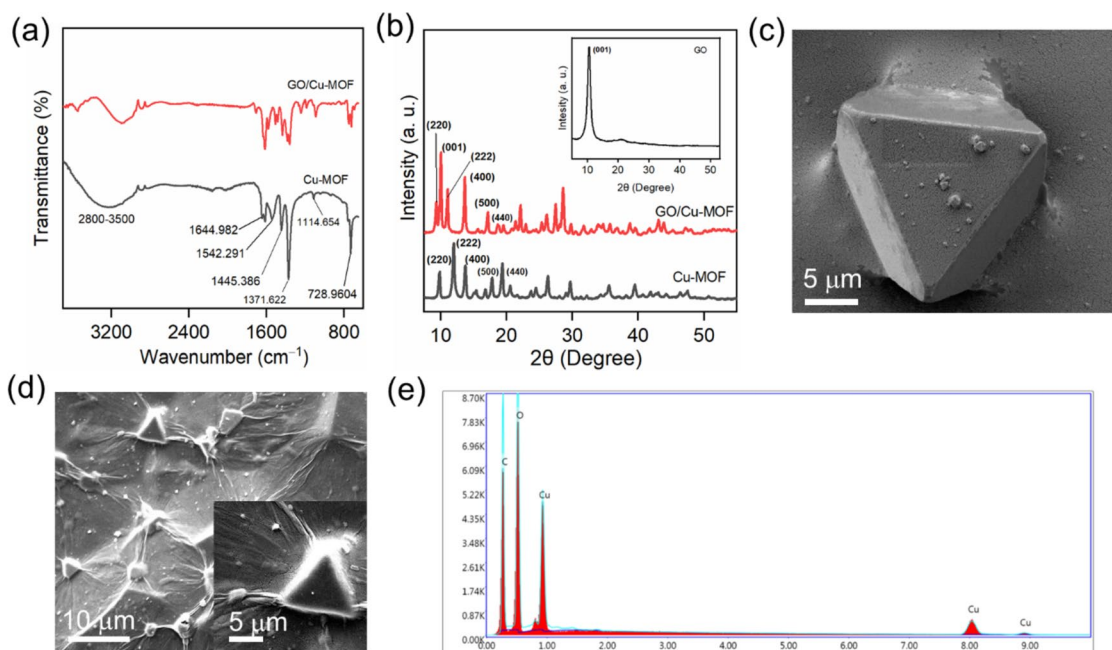


Figure 2. (a) FTIR (b) XRD spectra of Cu-MOF and GO/Cu-MOF. (c) SEM image of the Cu-MOF crystal and (d) SEM image of the GO/Cu-MOF/SPE. The inset represents the image at higher magnification. (e) Represent the EDAX analysis of the synthesized Cu-MOF material.

GO. Changes in intensity and peak positions in the composite pattern compared to pure Cu-MOF are typical for composite materials^{45,63}. In addition to the presence of the intense GO peaks at 12° indicate the successful integration of GO onto the Cu-MOF surface without disrupting the crystalline structure.

The investigation of changes in the carbon lattice structure of GO and GO/Cu-MOF nanosheets was carried out through Raman spectroscopy. In Fig. S1, the Raman spectra of the bare SPE reveal distinctive peaks at 1335 and 1568 cm⁻¹ corresponding to the D- and G-bands, respectively. Additionally, overtone peaks of the 2D band were identified at 2675 cm⁻¹⁷². The modifications with GO/Cu-MOF exhibited subtle variations in the intensity of the G, D, and 2D bands across all modified electrodes, likely due to differences in the degree of graphitization. A slight enhancement in the A_D/A_G ratio was observed in the GO/Cu-MOF compared to the bare electrode, indicating an increase in defect sites on the nanomaterial-modified electrode.

The morphological characteristics of the materials were investigated by SEM imaging. Figure 2c illustrates the structural configuration of the Cu-MOF crystal, displaying a distinctive condensed octahedral pattern. The pristine carbon SPE, GO/SPE (Fig. S2), and the GO/Cu-MOF (Fig. 2d) modified SPEs were also examined using SEM. In comparison to the pristine SPE carbon surface, the electrode modified with GO nanosheets (Fig. S2) showed a wavy, folded shaped thin layer, featuring interconnected network of stacked GO thin sheets on the working substrate. However, the SEM image of the GO/Cu-MOF composite surface (Fig. 2d) showed a homogeneously embedded MOF crystal within the GO sheets (Fig. 2d). To further analyse the elemental composition of the synthesized Cu-MOF crystal, energy-dispersive X-ray spectroscopy (EDX) was employed (Fig. 2e). The presence of copper (Cu), carbon (C) and oxygen (O) on the electrode indicates the elemental composition associated with the MOF, affirming the successful formation of Cu-MOF.

Electrochemical characterization of the GO/Cu-MOF modified electrode

Ensuring the electrochemical stability of electrodes modified with GO/Cu-MOF in PBS not only plays a pivotal role in shaping the outcomes of the biosensor but also significantly contributes to the overarching improvement in the efficiency of the system. Figure 3a shows the cyclic voltammograms (CVs) of bare SPE, GO, Cu-MOF and GO/Cu-MOF modified SPE recorded in the presence of 1 mM [Fe(CN)₆]^{3-/4-} in 0.01 M PBS at a scan rate of 0.05 V/s. Typical quasi-reversible voltammograms were obtained in all cases. The GO showed lower peak currents compared with the bare electrode due to the presence of the oxygen functionalities on its surface, which repels the redox anions. When the Cu-MOF was dropcasted on the electrode, a significant enhancement in the peak current were obtained indicating the increase in the electrocatalytic activity, faster electron transfer and higher surface area⁷³. However, the CV scans of the MOF modified electrode were not stable in PBS and a decrease in the current was observed over time. On the other hand, the GO/Cu-MOF/SPE showed a noticeable enhancement in the anodic and cathodic peak currents (i_{pa} : +21.7 μA and i_{pc} : -22.8 μA) compared to other modified electrodes as illustrated Fig. 3a. A decrease in the peak-to-peak separation (ΔE) was also observed. These results suggest that the composite-modified electrode has a higher surface area and a faster electron transfer rate compared to the electrode modified with the virgin MOF. This improvement is likely due to the synergistic effect of the two materials. Moreover, repetitive scans showed very good stability of the composite-modified electrode. In our

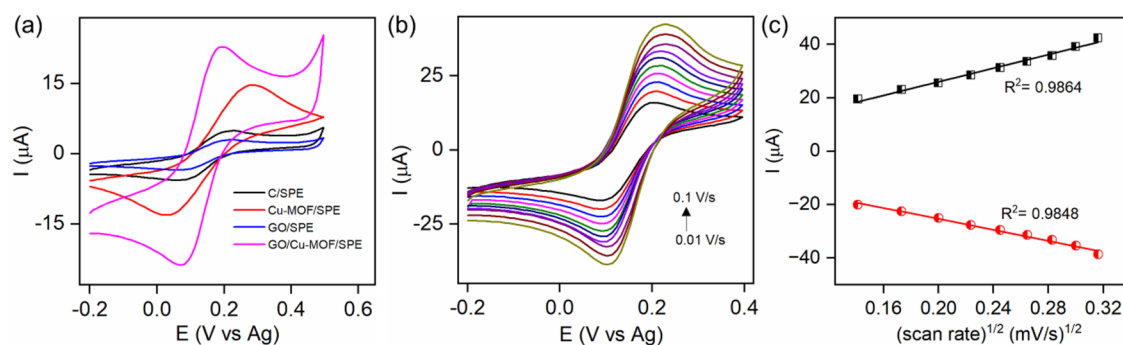


Figure 3. (a) CVs of the bare, GO, Cu-MOF, GO/Cu-MOF-modified electrodes at a scan rate of 0.05 V/s. (b) represent the scan rate effect on the CV of GO/Cu-MOF modified SPE. (c) Derivative plot of square root of scan rate vs current obtained from 2b. All the measurements were recorded in 0.01 M PBS containing 1 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$.

endeavor to understand the influence of scan rate on redox activity, we recorded CV curves for GO/Cu-MOF/SPE at varying scan rates ranging from 0.01 to 0.1 V/s, as depicted in Fig. 3b. The associated derivative linear plot (Fig. 3c) illustrates the cathodic/anodic peak current densities in correlation with the square root of the scan rate. This representation underscores that the primary mode of mass transport predominantly occurs through a diffusion-controlled process. The results of this in-depth investigation not only illuminate the electrochemical dynamics but also provide compelling evidence supporting the stability claim for the GO/Cu-MOF nanocomposite seamlessly integrated into the structural composition of flexible SPEs.

Electrochemical immunosensor studies

In line with “Preparation of the GO/Cu-MOF composite and coating of the electrodes” section of the experimental protocol, pyrene linker with a terminal carboxyl group was introduced onto the GO/Cu-MOF electrode to enable the efficient immobilization of monoclonal antibodies (mAbs). The PY linker attaches to the GO through π - π stacking interactions. The terminal carboxyl groups of the linker are then activated by EDC, which forms a reactive intermediate stabilized by NHS. This intermediate reacts with the amine groups on the antibodies, resulting in the formation of stable amide bonds that ensure strong attachment. This enhances the functionality and stability of the biosensor. Figure 1B,C illustrates the modification of two distinct working substrates (WE1 and WE2) with different mAbs, specifically, mouse anti-*Mycobacterium pneumoniae* (anti-*M. p.*) and anti-*Legionella pneumophila* (anti-*L. p.*) monoclonal antibodies. Diverging from conventional single working electrodes, the utilization of a dual SPE offers the advantage of simultaneous detection of two different analytes within the same sample. The measurement of electrochemical signals and subsequent data processing can be executed sequentially. Upon the specific interaction of target antigens with the surface of a mAb-modified sensor element, an insulating layer resulted due to the immune complex. This binding creates a barrier to the electron-transfer process at the electrode-electrolyte interface and the change in the electrochemical signal can be correlated with the concentrations of the target analyte.

Figure 4 showcases the electrochemical characterization during various stages of the *M. pneumoniae* and *L. pneumophila* biosensor fabrication on the GO/Cu-MOF modified SPE surface. The CV measurements (Fig. 4a,b) for the GO/Cu-MOF/SPE are presented both before and after each modification step. The GO/Cu-MOF/SPE displayed a quasi-reversible redox peak with a peak separation (ΔE_p) of approximately 0.11 V, indicative of enhanced electron transfer at the electrode-electrolyte interface. Upon integrating PY onto the electrode, a slight reduction in the peak current was noted, indicating the π - π stacking interacting of the PY linker on the composite showing terminal acidic carboxy groups^{74,75}. Subsequently, after activation with EDC-NHS and incubation with the antibodies, a significant decrease in both anodic and cathodic peak currents was observed, accompanied by a slight increase in ΔE . This suggests a deceleration in the electron transfer rate, likely due to the hindering effect of the monoclonal antibodies. Furthermore, the following incubation with BSA led to a subsequent reduction in peak current and an increase in ΔE . The observed effect can be attributed to the blocking of the remaining free surface of the modified electrode by BSA to minimize the non-specific adsorption. Subsequently, following the *M. pneumoniae* and *L. pneumophila* antigens binding on the immunosensor, a substantial difference in the current is evident, enabling specific antigen detection.

It is crucial to investigate the binding duration of the antigens on the dual electrochemical immunosensor to achieve the maximum binding signal. The optimization of binding time involved incubating the immunosensor with *M. pneumoniae* and *L. pneumophila* antigens for various intervals (ranging from 0 to 60 min) on the customized GO/Cu-MOF/PY/EDC-NHS/anti-(*M. p.* & *L. p.*) electrode surfaces, respectively. CVs were recorded after each incubation period, revealing an increase in immunosensor response with prolonged incubation time, as clearly demonstrated in Fig. S3.

The optimal immunosensor response for *M. pneumoniae* and *L. pneumophila* was attained at 30 min. Although the response signal increased up to 60 min, there was not a substantial difference in the current response. In pursuit of proof-of-concept, we conducted DPV studies (Fig. 4b,d) under the aforementioned conditions, strategically leveraging the well-documented redox behaviour of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in the PBS buffer. In this context,

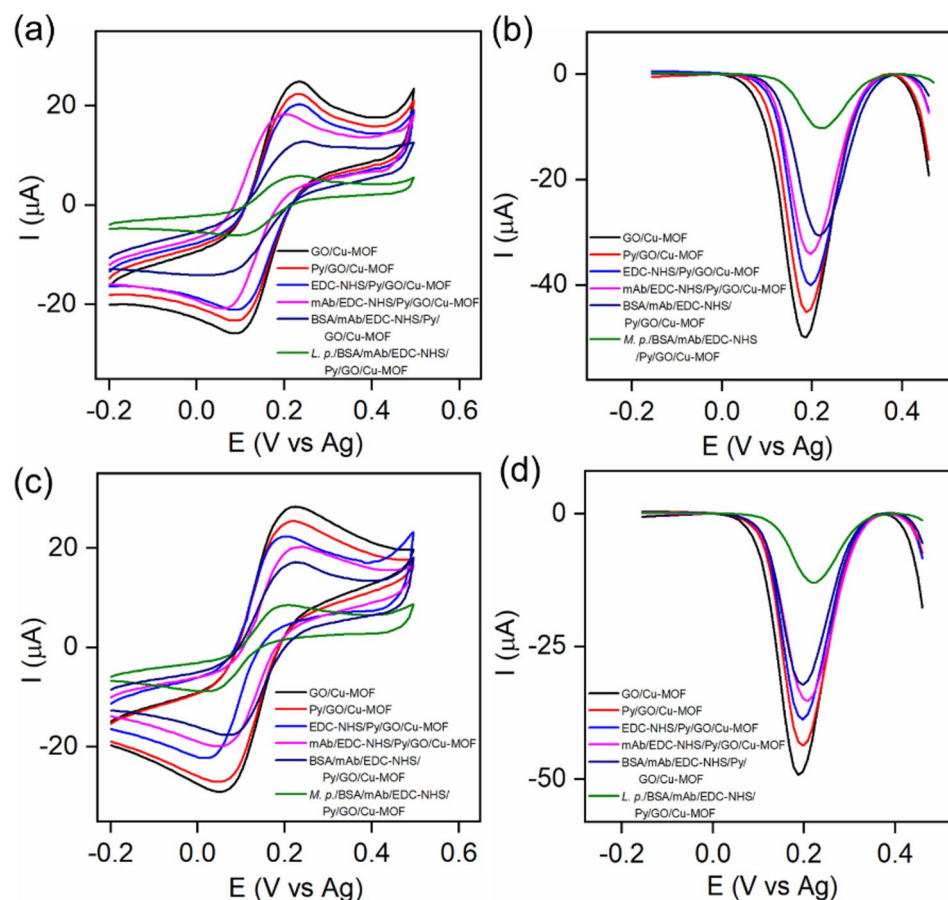


Figure 4. The electrochemical characterization of the immobilization of the *M. pneumoniae* and *L. pneumophila* antigens using (a,c) cyclic voltammetry and (b,d) Differential pulse voltammetry in 1 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox mediator in 0.01 M PBS (pH 7.4) electrolyte.

the DPV results also exhibited a parallel trend, marked by more substantial variations in the reduction peak current, thus corroborating the observations from the CV analysis.

The comprehensive DPV analysis, as depicted in Fig. 5a,c, elucidates the immunosensor's performance on the GO/Cu-MOF/PY/EDC-NHS/anti-(*M. p.* & *L. p.*) electrodes across a range of *M. pneumoniae* and *L. pneumophila* antigen concentrations (0, 1, 10, 50, 100, 500 pg/mL and 1, 10, 100 ng/mL) in PBS. The cathodic peak ($E_{pc} = 0.12$ V), linked to the known redox mediator $[\text{Fe}(\text{CN})_6]^{3-/4-}$, revealed a minimum current response of 13.3 μA when the immunosensor was incubated in 100 ng/mL concentration of *M. p.*, signifying heightened immune complex formation on the immunosensor. Noteworthy changes in the cathodic peak current were observed with varying analyte concentrations, discerned in the linear derivative plot illustrating current changes (Fig. 5b). A linear fit for the DPV results, expressed as $\Delta I = (I_0 - I_t/I_0)$, where I_0 is the absolute peak current value of the immunosensor tested in PBS without antigens and I_t is the absolute peak current value of the immunosensor after incubation with the desired antigen concentration in PBS buffer, resulted in a linear equation of $\Delta I = 38.1937 + 12.0525 \log C_{M.p.}$ with a correlation coefficient of 0.9893. Given the DPV technique and the studied *M. p.* antigen concentration range, the detection limit was calculated as 9.4 pg/mL.

Similarly, the DPVs recorded from the other electrode surface of the immunosensor GO/Cu-MOF/PY/EDC-NHS/anti-*L. p.* and tested with varied concentrations of *L. p.* antigens in PBS are illustrated in Fig. 5c. The anti-*L. pneumophila* modified sensor platform demonstrated a more pronounced correlation between the change in current and analyte concentrations. The derivative plot (Fig. 5d) elucidates that among the studied concentrations, the immunoelectrode treated with a 100 ng/mL sample exhibited the lowest peak current of -12.6 μA , indicating a concentration-dependent linear voltammetric response. The linear regression equation $\Delta I = 36.088 + 10.518 \log C_{L.p.}$ with an R^2 of 0.9848. The detection limit was determined to be 8.3 pg/mL.

The investigation into the immunosensor's reproducibility involved conducting triplicate experiments for each protein as shown in Fig. S4. The relative standard deviations for these measurements fell within the range of 2.4 to 6.5%, signifying an excellent level of reproducibility in the immunosensor's performance.

The dual electrochemical immunosensor's selectivity was assessed against other related pathogens, including canine adenovirus 1 (CAV1), human metapneumovirus (hMPV) B, Influenza A (Cambodia) virus (IAV), Influenza B (Austria) virus (IBV), and respiratory syncytial virus B (RSVB) Lysate antigens to confirm the specificity

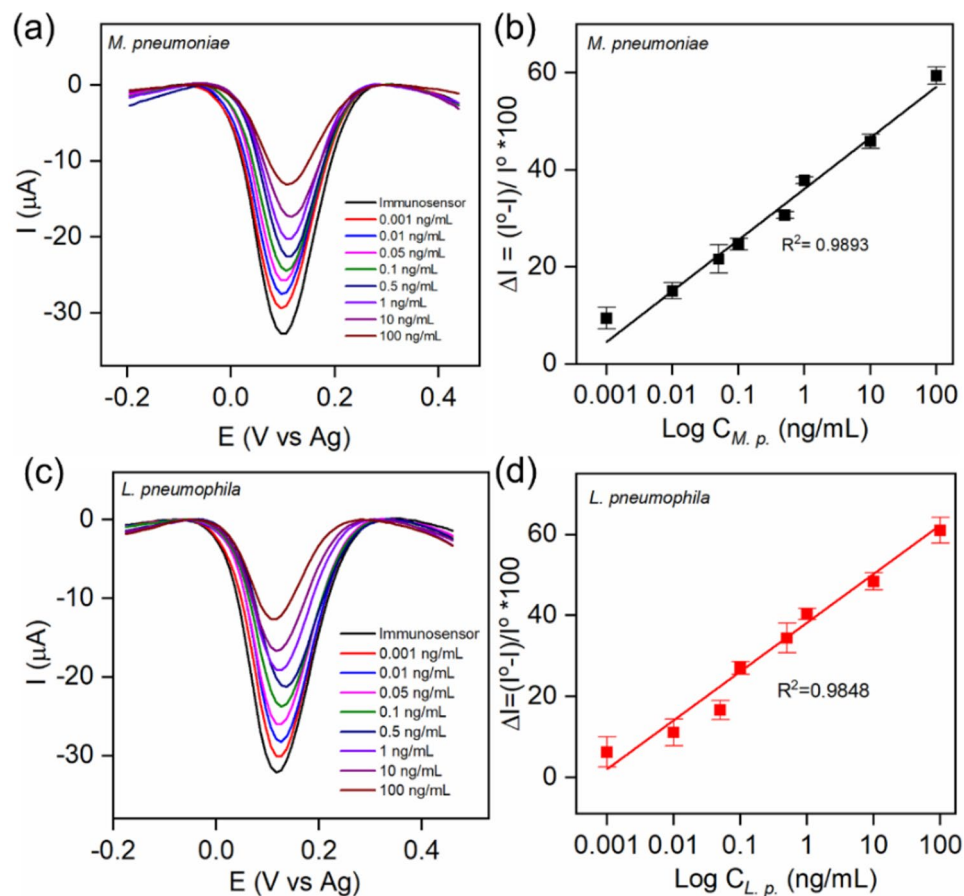


Figure 5. (a) DPVs of GO/Cu-MOF/PY/EDC-NHS/anti-*M. p.* electrodes against various concentrations of antigen *M. p.* and (b) the calibration curve derived from the DPVs expressed in ΔI . The error bars represent the standard deviations of triplicate measurements.

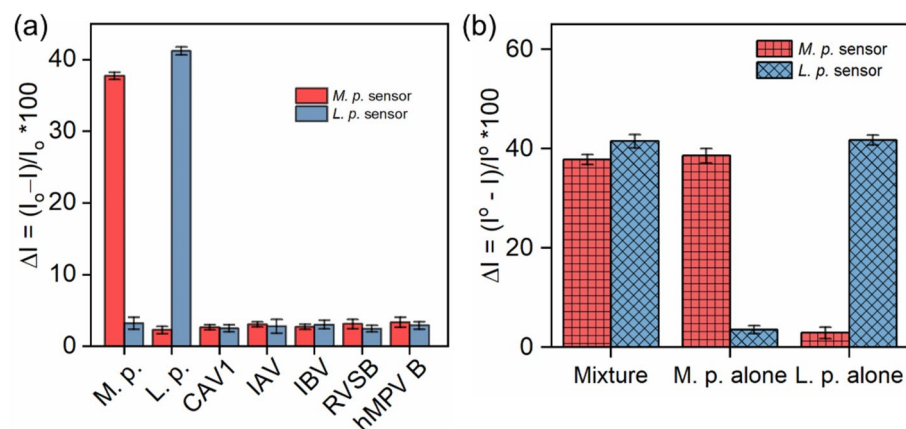


Figure 6. (a) The response of the dual immunosensor to 1 ng/mL of *M. pneumoniae* (*M. p.*), *L. pneumophila* (*L. p.*), and 10 ng/mL of CAV1, hMPV B, IAV, IBV, RSVB antigens. (b) Immunosensor responses for the concurrent detection of *M. p.* and *L. p.* both in a mixture and individually. The error bars represent the standard deviations of triplicate measurements.

Analyte	Spiked concentration (ng/mL)	Amount found (ng/mL)	Recovery %	RSD% (n = 3)
<i>M. pneumoniae</i>	0.1	0.105	105	4.1
	1	1.02	102	4.9
	10	10.37	103.7	3.2
<i>L. pneumophila</i>	0.1	0.098	98	3.1
	1	1.03	103	4.84
	10	10.26	102.6	3.01

Table 1. The recovery studies of *M. pneumoniae* and *L. pneumophila* spiked in tap water.

of the immunosensor response¹⁹. The immunosensor was individually incubated with the selected pathogens *M. pneumoniae* and *L. pneumophila*, as well as the interferents, and the response was recorded after washing with PBS buffer. As illustrated in Fig. 6a, the immunosensor's response to specific proteins was significantly higher than that to non-specific proteins, indicating the method's appropriate selectivity.

Likewise, as-developed electrochemical immunosensor platform were further analyzed for cross-hybridization within the selected pathogen and the relevant results are furnished in Fig. 6b. From the DPV derived ΔI value represented in histogram, it is again evident that the constructed immunosensor platform exhibiting selective current against the specific target. As can be seen, when the two antigens were mixed, high responses were obtained for the two electrodes on the biosensor, whereas only one sensor showed high signal when incubated with the specific antigen, implying the feasibility of applying our immunosensor for multiplexed detection of these pathogens.

Table S1 presents a comparative analysis of the analytical performance of the newly introduced electrochemical immunosensor alongside similar and alternative sensor methodologies documented in the literature. This assessment underlines a distinct focus on the detection of entire bacterial pathogens rather than their protein forms in many existing sensor platforms. Furthermore, it is noteworthy that the developed dual electrochemical immunosensor boasts several compelling advantages over other immunosensor designs. Specifically, it achieves a notably low detection limit (*M. pneumoniae* 9.4 pg/mL and *L. pneumophila* 8.3 pg/mL) and the analytical response time of 30 min. stands out as significantly shorter than those reported in other reported methods.

To assess the practical utility of the newly developed electrochemical immunosensor system in monitoring the bacterial pathogens in tap water, we conducted experiments involving the spiking of various concentrations of *M. pneumoniae* and *L. pneumophila* antigens into tap water samples. As illustrated in Fig. S5, slight variations in the current response were observed for both samples. Notably, Table 1 shows the recovery percentages for the immunosensor to both antigens ranged from 98 to 105%, underscoring the system's robustness and potential for on-field detections. It is essential to highlight that the conventional gold standard method for identifying the *pneumophila* antigen demands a considerable timeframe of up to 10 days. Consequently, the developed dual electrochemical immunosensor platform emerges as an attractive alternative analytical solution, providing rapid screening capabilities in 30 min for contaminated water samples.

Conclusions

A novel dual immunosensor platform was developed for the detection of *Mycoplasma pneumoniae* and *Legionella pneumophila* antigens in water, capitalizing on the distinctive characteristics of GO/Cu–MOF nanostructures. The synergistic interactions between the GO and Cu–MOF led to improved redox behaviour, stability and conductivity. The successful functionalization of the composite surface has led to the efficient immobilization of the antibodies, facilitating the robust formation of the immune complexes with the target antigens. The detection was realized by measuring the differential pulse voltammetric signals of the immunosensors in ferro/ferricyanide redox probe. The use of monoclonal antibodies allowed the specific and simultaneous detection of *M. pneumoniae* and *L. pneumophila* antigens. The designed dual immunosensor is straightforward to construct and exhibits attributes such as high sensitivity, selectivity, rapid response time, and reproducibility. Considering the urgent need for the prompt detection of *pneumonia* pathogens and their associated complications, a straightforward and sensitive immunoassay proves highly advantageous. Further refinements to the developed immunosensor design could enhance its capabilities for the multiplexed detection and differentiation of various respiratory pathogens for clinical and environmental applications.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

K. P.: Data curation, Formal analysis, Methodology, Writing—original draft. R. E.: Formal analysis, Methodology, Writing—original draft. M. M.: Supervision, Writing—review & editing. S. E.: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing—review & editing.

Competing interests

The authors declare no competing interests.

Additional information

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