



Cite this: *Sens. Diagn.*, 2025, 4, 803

Ultrasensitive electrochemical detection of methotrexate in biological fluids using NiMn₂O₄/CNT nanocomposite-modified electrode†

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Methotrexate (MTX) is a widely used chemotherapeutic drug with a narrow therapeutic index, making its precise monitoring crucial for effective treatment and minimizing side effects. This study focuses on the development of a clinically applicable NiMn₂O₄/CNT nanocomposite-modified glassy carbon electrode (NiMn₂O₄/CNT-GCE) for the sensitive and selective electrochemical detection of MTX. The NiMn₂O₄ nanomaterial was synthesized via a co-precipitation method followed by calcination, and its composite with CNTs was optimized to enhance electrochemical performance. The sensor demonstrated a detection limit as low as 0.627 nM and a broad linear detection range (0.05–3 μM), attributed to the synergistic effects of NiMn₂O₄ and CNTs that enhance electron transfer and active site availability. Moreover, the NiMn₂O₄/CNT-GCE was successfully applied to detect MTX in spiked serum and urine samples, achieving recovery rates of 96–99% with relative standard deviations below 3.5%. Its minimal interference with common metabolites and excellent stability makes it ideal for therapeutic drug monitoring. This work underscores the potential of NiMn₂O₄/CNT as a promising platform for real-time clinical diagnostics and advanced electrochemical sensing applications.

Received 9th May 2025,
Accepted 10th July 2025

DOI: 10.1039/d5sd00064e

rsc.li/sensors

1. Introduction

Methotrexate (MTX) is a widely used chemotherapeutic and immunosuppressive drug, extensively employed in the treatment of cancers such as leukemia¹ and autoimmune diseases,² including rheumatoid arthritis.^{3–5} Despite its therapeutic benefits, MTX poses significant risks due to its narrow therapeutic index.⁶ Excessive dosages can lead to severe toxic side effects,⁷ including hepatotoxicity and bone marrow suppression, while sub-therapeutic levels may result in reduced efficacy.^{8,9} Consequently, precise and reliable monitoring of MTX levels in biological fluids is essential to ensure patient safety and optimize therapeutic outcomes.^{10–13}

Conventional techniques for MTX detection, such as high-performance liquid chromatography (HPLC),^{14,15} enzyme-linked immunosorbent assays (ELISA),^{16,17} and mass spectrometry,¹⁸ are well-established for their sensitivity and specificity.¹⁸ However, these methods are often associated with limitations, including high operational costs, labor-intensive sample preparation, and the requirement for sophisticated

instrumentation.^{19–21} Given these challenges, electrochemical sensors offer a compelling alternative due to their simplicity, rapid response times, and exceptional sensitivity, making them ideal for real-time drug monitoring.^{22–25}

The performance of electrochemical sensors heavily depends on the electrode material, which plays a pivotal role in enhancing electrocatalytic activity and stability.^{26,27} Nickel manganese oxide (NiMn₂O₄), a spinel-type oxide, has garnered significant attention in electrochemical applications due to its unique properties, including high electrical conductivity, multiple redox states, and synergistic interactions between nickel and manganese ions.^{28–32} Despite these advantages, standalone NiMn₂O₄ often suffers from moderate conductivity and limited surface area, which can hinder its practical application.

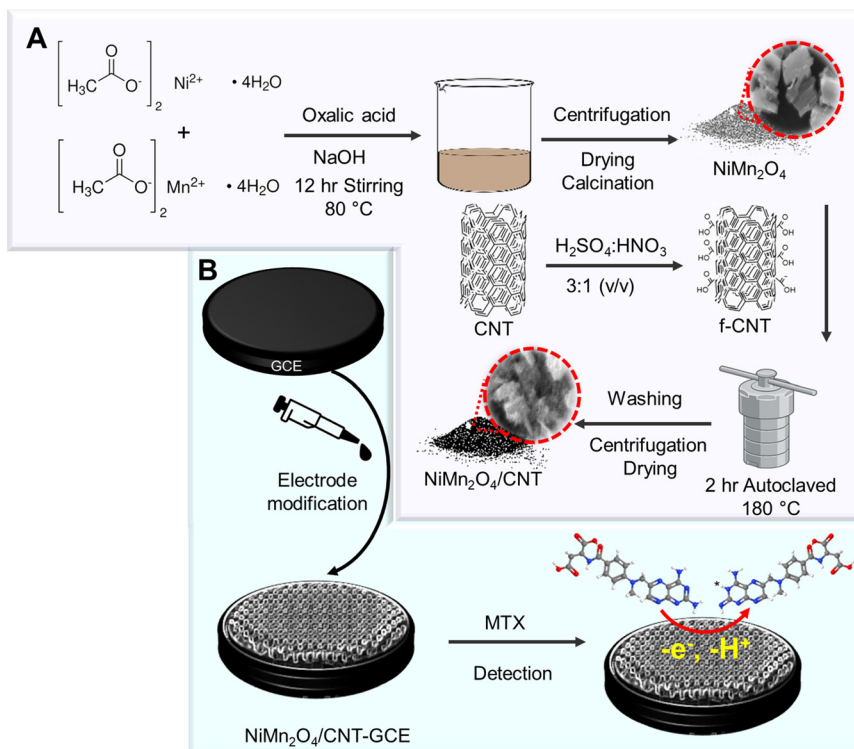
To address these limitations, NiMn₂O₄ can be combined with carbon nanotubes (CNTs), which serve as an excellent supporting matrix due to their remarkable electrical conductivity, high surface area, and mechanical stability.^{33,34} The integration of NiMn₂O₄ and CNTs into a composite material leverages the complementary properties of both components, providing a synergistic effect that enhances electron transfer, increases active sites, and improves overall sensor performance.³⁵

In this study, we employed a two-step approach to develop a highly sensitive electrochemical sensor for MTX detection (Scheme 1). In the first step, a NiMn₂O₄/CNT nanocomposite

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† Electronic supplementary information (ESI) available: Supplementary data associated with this article can be found in the online version. See DOI: <https://doi.org/10.1039/d5sd00064e>





Scheme 1 (A) Schematic representation of the two-step synthesis process for the NiMn₂O₄/CNT composite: co-precipitation of NiMn₂O₄ followed by hydrothermal treatment with functionalized CNTs. (B) Fabrication of the NiMn₂O₄/CNT-modified glassy carbon electrode (NiMn₂O₄/CNT-GCE) and its application for the electrochemical detection of methotrexate (MTX).

was synthesized using a co-precipitation method followed by calcination³⁶ and hydrothermal treatment with functionalized CNTs to optimize the composite's electrochemical properties.^{37,38} In the second step, the nanocomposite was used to fabricate a modified glassy carbon electrode (NiMn₂O₄/CNT-GCE), which was applied for the electrochemical detection of MTX. This systematic workflow ensured the enhancement of both material properties and sensor performance.

The NiMn₂O₄/CNT composite and its modified electrode were systematically characterized using X-ray diffraction (XRD), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), while the electrochemical performance was evaluated using cyclic voltammetry (CV) and differential pulse voltammetry (DPV). The sensor demonstrated remarkable sensitivity and selectivity, achieving a detection limit as low as 0.627 nM and a wide linear range of 0.05–3 μM.

The practical applicability of the sensor was validated through the detection of MTX in spiked serum and urine samples, achieving recovery rates of 96–99% with relative standard deviations below 3.5%. These results highlight its potential for clinical diagnostics. To the best of our knowledge, this study represents the first application of a NiMn₂O₄/CNT composite for MTX detection, offering a novel contribution to the field of electrochemical sensing and paving the way for real-time therapeutic drug monitoring.

2. Experimental

2.1. Chemicals

The materials and reagents used in this synthesis were of analytical grade and sourced from Sigma Aldrich. All chemicals were employed directly without further purification. The following were utilized: nickel(II) acetate tetrahydrate (Ni(CH₃COO)₂·4H₂O, 99.99% purity), oxalic acid (C₂H₂O₄), manganese(II) acetate tetrahydrate (Mn(CH₃COO)₂·4H₂O, 99.99% purity), sodium hydroxide (NaOH), and ethanol (C₂H₅OH, 99.08% purity). Human serum was purchased from Sigma-Aldrich and used as received. Human urine samples were voluntarily provided by a healthy adult donor from the research group with informed consent. The samples were anonymized and used solely for research purposes in accordance with institutional ethical guidelines. Given the non-invasive nature of the sample collection and lack of identifying information, IRB approval was not required. A phosphate buffer solution (0.05 M PBS) was prepared by dissolving the appropriate amounts of disodium hydrogen phosphate (Na₂HPO₄) and monosodium hydrogen phosphate (NaH₂PO₄) in deionized (DI) water. For electrode preparation, carbon black was used. Commercially purchased multi-walled carbon nanotubes (CNTs) with a diameter of 1–2 nm and a length of approximately 50 μm were also included. Deionized water was used as the solvent throughout the study.



2.2. Instrumentation

To thoroughly understand the structure and properties of the synthesized catalysts, a variety of characterization techniques were employed. XRD analysis, performed using a Rigaku MiniFlex600 (Japan), provided insight into the crystal structure of the materials. XPS, conducted with a K-Alpha (Thermo Fisher Scientific, USA), verified the chemical composition of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite, specifically examining the binding energies of key elements such as nickel, oxygen, and carbon. SEM using a ZEISS Sigma 500 (Germany) offered an initial view of the surface morphology and microstructure. For higher-resolution imaging, field emission scanning electron microscopy (FE-SEM; TESCAN MIRA LMH, Czech Republic) was performed to provide detailed surface and structural insights. TEM (JEOL JEM-1010, Japan) offered a deeper understanding of the nanocomposite's intricate structural features.

The electrochemical properties of the materials were evaluated using a CHI 660D electrochemical workstation (CH Instruments, USA) in a standard three-electrode configuration. In this setup, a platinum wire served as the counter electrode, an Ag/AgCl electrode was used as the reference electrode, and the working electrode was either a bare GCE (diameter of 3.0 mm) or one modified with the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite.

Electrochemical impedance spectroscopy (EIS) was performed to assess the interfacial charge transfer properties of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ -modified electrode. The measurements were conducted over a frequency range of 1 Hz to 100 kHz, with an AC amplitude of 5 mV at an open circuit potential (OCP) of 0.267 V for bare GCE, 0.247 V for CNT-GCE, 0.250 V for NiMn_2O_4 -GCE, and 0.249 V for $\text{NiMn}_2\text{O}_4/\text{CNT}$ -GCE.

2.3. Synthesis of NiMn_2O_4

NiMn_2O_4 was synthesized using a co-precipitation method. Initially, 100 mL of a solution containing 0.025 M nickel acetate tetrahydrate ($\text{Ni}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$) and 0.05 M manganese(II) acetate tetrahydrate ($\text{Mn}(\text{CH}_3\text{COO})_2 \cdot 4\text{H}_2\text{O}$) was prepared by dissolving the precursors in a 1 : 1 volume ratio of DI water and ethanol. The two solutions were mixed thoroughly to obtain a clear solution. Subsequently, 5 g of oxalic acid crystals were added, and the mixture was stirred at 700 rpm for 15 minutes at room temperature to ensure homogeneity.

Next, 100 mL of 1 M NaOH was slowly added to the solution, followed by continuous stirring for 12 hours at 80 °C. Upon completion, the resulting solution was cooled to room temperature, and the precipitate was collected *via* centrifugation at 8000 rpm. The precipitate was washed several times with ethanol and water to remove impurities, then dried overnight in a vacuum oven at 60 °C. Finally, the dried material was calcined at 450 °C for 2 hours to achieve the desired crystalline phase of NiMn_2O_4 .³⁶

2.4. Synthesis of $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite

The synthesis of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite involved the acid treatment of pristine CNTs. Specifically, 0.1 g of

pristine CNTs was dispersed in a mixture of nitric acid (HNO_3) and sulfuric acid (H_2SO_4) at a volume ratio of 1 : 3 in 80 mL of DI water. The resulting mixture underwent ultrasonication for 4 hours to facilitate functionalization. After this period, the mixture was cooled to room temperature. The acid-treated CNTs were thoroughly washed with DI water until a neutral pH was achieved and then dried overnight in a vacuum oven at 60 °C. This acid treatment introduces carboxyl ($-\text{COOH}$) groups onto the CNT surface, which enhances their dispersion in aqueous media and promotes better interfacial interaction with metal oxides. These surface modifications improve the electron transfer properties of the resulting nanocomposite, thereby contributing to the improved electrochemical performance of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ sensor.

For the nanocomposite synthesis, 16.6 mg of the acid-treated CNTs was added to a beaker containing 10 mL of DI water. Separately, 225 mg of the prepared NiMn_2O_4 was dispersed in 10 mL of DI water. This ratio ($\text{NiMn}_2\text{O}_4 : \text{CNT} = \sim 13.6 : 1$, w/w) was selected based on preliminary trials that showed optimal electrochemical performance in terms of current response and stability. While other ratios were briefly explored (*e.g.*, 5 : 1 and 20 : 1), the selected formulation provided the best balance between conductivity and catalytic activity. The selected ratio was deemed sufficient for demonstrating the sensor's analytical performance, which is the primary focus of this study. The two solutions were combined and mixed thoroughly with constant stirring at 700 rpm for 20 minutes, followed by ultrasonication for 10 minutes to ensure proper dispersion. The resulting mixture was transferred into an autoclave and heated at 180 °C for 3 hours. After cooling to room temperature, the product was washed multiple times with ethanol and DI water to remove residual impurities. The final black precipitate of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite was dried overnight in a vacuum oven at 70 °C.³⁷

2.5. Preparation of $\text{NiMn}_2\text{O}_4/\text{CNT}$ -GCE electrode

To prepare a clean surface for the GCE prior to modification, the GCE was polished sequentially using alumina slurries with progressively smaller particle sizes of 0.3 μm , 0.1 μm , and 0.05 μm . This gradual polishing process aimed to create a smooth and contaminant-free surface for electrode modification. Following the polishing process, the GCE was thoroughly cleaned by sonicating it repeatedly in ethanol and DI water to remove any residual alumina particles and contaminants. Next, a stable suspension for electrode modification was prepared by dispersing 2 mg of the synthesized $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite in 1 mL of DI water. This suspension was homogenized through vortex mixing and ultrasonication to ensure uniform distribution of the nanocomposite. Subsequently, 8 μL of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ suspension was applied onto the cleaned GCE surface and dried using an infrared lamp. The modified electrode, designated as



NiMn₂O₄/CNT-GCE, was employed for the electrochemical detection of MTX in this study.

3. Results and discussion

3.1. Characterization of NiMn₂O₄/CNT nanocomposite

The structural and morphological properties of the NiMn₂O₄/CNT nanocomposite were comprehensively analyzed using XRD, SEM, TEM, and XPS techniques.

The XRD patterns (Fig. 1A) confirmed the formation of the spinel NiMn₂O₄ phase, with diffraction peaks at 2θ values of 17.7°, 29.8°, 35.2°, 36.9°, 43.0°, 53.2°, 56.9°, 62.6°, 74.1°, and 75.1°, corresponding to the (111), (220), (311), (222), (400), (422), (511), (440), (533), and (622) planes, respectively. These peaks match the reference pattern from JCPDS card no. 84-0542 (space group: $R\bar{3}$), verifying the successful synthesis of pure-phase NiMn₂O₄. The composite exhibited a broad peak at approximately 21.4°, attributed to the (002) plane of CNTs, confirming their successful integration. The (002) plane of CNTs enhances the composite's electrical conductivity, facilitating efficient electron transfer and providing a stable support network. This integration ensures the structural integrity and functional efficiency of the composite, making it particularly suitable for electrochemical applications.

The chemical composition and oxidation states of the NiMn₂O₄/CNT nanocomposite were investigated using XPS (Fig. 1B–F). The survey spectrum (Fig. 1B) revealed the presence of Ni, Mn, O, and C, confirming uniform elemental distribution. High-resolution XPS spectra identified multiple oxidation states for Mn and Ni, which are crucial for redox behavior. The Mn 2p spectrum (Fig. 1C) displayed peaks at 641.7 eV and 644.3 eV for Mn³⁺ and at 653.46 eV and 655.5 eV for Mn⁴⁺, underscoring their role in enabling efficient redox reactions. Similarly, the Ni 2p spectrum (Fig. 1F) showed peaks at 854.85 eV and 872.4 eV for Ni²⁺, and 856.15 eV and 873.86 eV for Ni³⁺, along with satellite peaks at 861.37 eV and 880.42 eV. The O 1s spectrum

(Fig. 1D) revealed three oxygen species: lattice oxygen (530.09 eV), oxygen vacancies (531.5 eV), and adsorbed water molecules (534.01 eV). These oxygen species play a pivotal role in facilitating redox reactions by providing active sites for electron transfer. The C 1s spectrum (Fig. 1E) confirmed successful hybridization with CNTs, showing peaks at 284.8 eV (C–C/C=C), 286.07 eV (C–O), and 289.18 eV (O–C=O).

The surface morphology of the synthesized materials was analyzed using SEM, as shown in Fig. 2. The SEM images of NiMn₂O₄ in Fig. 2A and B at different magnifications exhibit rectangular cube-like structures with a porous surface, which can facilitate ion diffusion and improve the electrochemical activity by providing more accessible pathways for electrolyte penetration. This porosity is likely due to CO₂ release during the calcination process. In the SEM image (Fig. 2C) of NiMn₂O₄/CNT, CNTs are observed coating the rectangular NiMn₂O₄ cubes, indicating successful hybridization. Elemental mapping analysis (Fig. 2D–H) confirms the uniform distribution of Ni, C, Mn, and O within the NiMn₂O₄/CNT composite. This uniform dispersion ensures structural integrity and facilitates consistent electron transfer pathways, thereby supporting stability and enhancing the electrochemical performance of the electrode material. While nanocubic structures are advantageous for consistent diffusion paths and uniform active sites, the rough surface morphology observed here provides increased surface roughness, potentially enhancing the exposure of active sites and improving electrochemical performance.

To analyze the detailed morphological features of the synthesized materials, TEM analysis was performed. Fig. 3A and B present TEM images of the NiMn₂O₄/CNT nanocomposite, showing well-defined nickel manganese oxide-carbon nanotube structures. The images highlight how CNTs are effectively attached to and interacting with the NiMn₂O₄ nanoparticles, indicating a strong interaction that enhances conductivity and stability. High-resolution TEM (HRTEM) in Fig. 3C reveals distinct lattice fringes, with

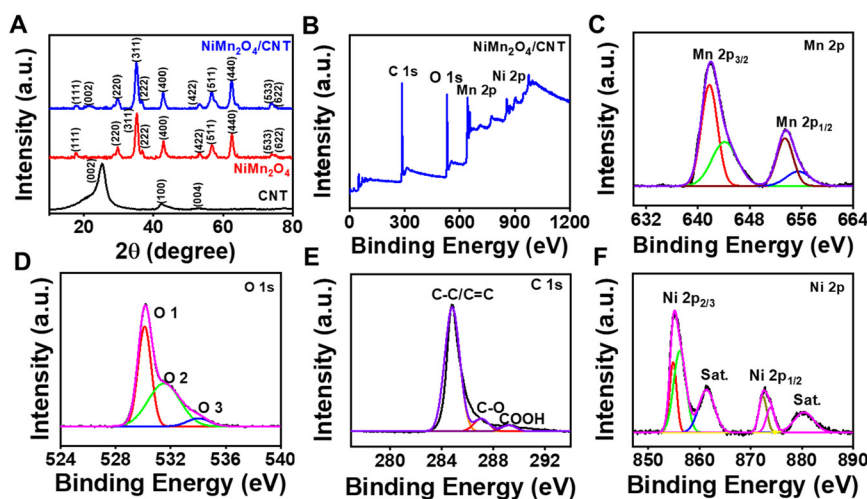


Fig. 1 (A) XRD patterns of CNT, NiMn₂O₄, and NiMn₂O₄/CNT. (B) XPS survey spectrum of NiMn₂O₄/CNT. (C–F) High-resolution XPS spectra of NiMn₂O₄/CNT: (C) Mn 2p, (D) O 1s, (E) C 1s, and (F) Ni 2p.



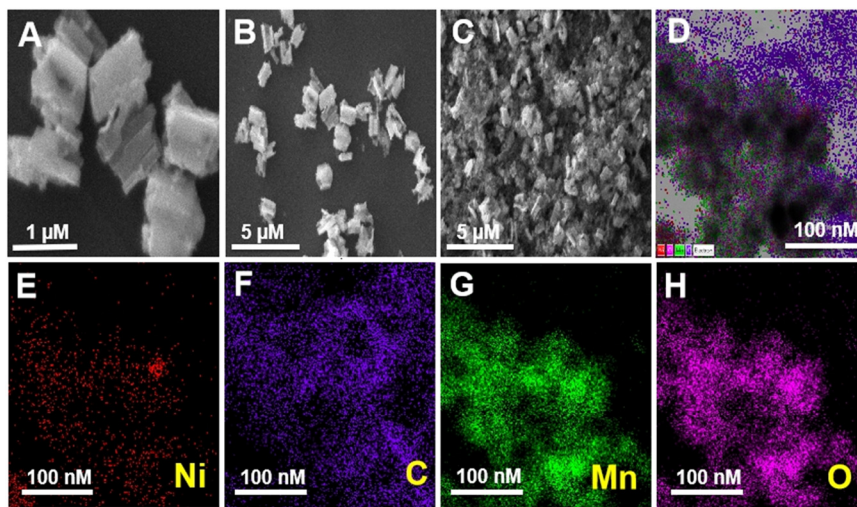


Fig. 2 SEM images of (A and B) NiMn_2O_4 at different magnification (C) $\text{NiMn}_2\text{O}_4/\text{CNT}$. (D–H) Elemental mapping analysis of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite showing elements Ni, C, Mn, and O.

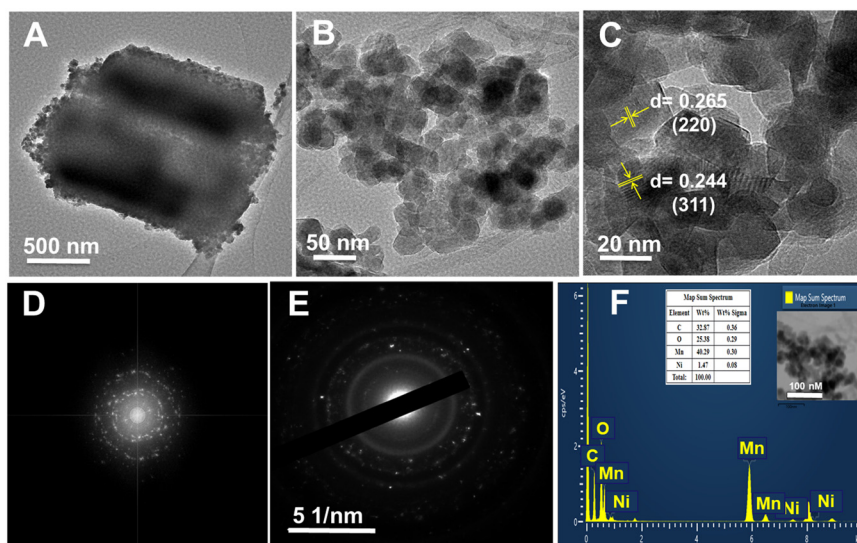
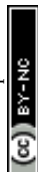


Fig. 3 (A and B) TEM image of $\text{NiMn}_2\text{O}_4/\text{CNT}$ (C) HR-TEM of $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite. (D) FFT pattern of $\text{NiMn}_2\text{O}_4/\text{CNT}$, (E) SAED pattern and (F) EDS analysis of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite (inset: corresponding TEM image of the analyzed area and the EDS elemental composition table).

measured interplanar spacings of approximately 0.244 nm and 0.265 nm. These spacings correspond to the (311) and (220) crystal planes of NiMn_2O_4 , which are critical for facilitating efficient electron transport and enhancing the material's stability during electrochemical processes. The observed crystallinity directly supports the nanocomposite's effectiveness in sensing applications. These spacings are critical as they correspond to the (311) and (220) crystal planes of NiMn_2O_4 , confirming the material's high degree of crystallinity, which is essential for facilitating efficient electron transport and enhancing electrochemical performance. These spacings correspond to the (311) and (220) crystal planes of NiMn_2O_4 , consistent with the XRD data at 2θ values of 35.2° and 29.8° , respectively. This confirms the crystallinity of the material. The FFT pattern in Fig. 3D

illustrates the crystalline order of the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite, further supporting the observed lattice fringes. Meanwhile, the selected area electron diffraction (SAED) pattern shown in Fig. 3E demonstrates concentric rings interspersed with bright spots, highlighting the polycrystalline nature of the NiMn_2O_4 material. These diffraction rings closely match the XRD peaks, further confirming the integration of NiMn_2O_4 nanocubes with CNTs. Additionally, the SAED pattern confirms the presence of the (111), (311), and (400) crystal planes, aligning with the XRD data for NiMn_2O_4 . This high degree of crystallinity is crucial for enhancing the electrochemical properties of the nanocomposite.

To further confirm the composition, EDS analysis was performed (Fig. 3F). The EDS spectrum reveals distinct peaks



corresponding to Ni, Mn, O, and C, verifying the elemental composition of the NiMn₂O₄/CNT nanocomposite. This analysis confirms the successful integration of NiMn₂O₄ and CNT components, which is essential for maintaining uniform active sites and ensuring consistent electron transfer pathways. Such uniformity supports structural stability and enhances the nanocomposite's efficiency in electrochemical sensing applications.

3.2. Electrochemical behavior of NiMn₂O₄/CNT-GCE

The electrochemical performance of the modified electrodes was evaluated using CV and EIS. As shown in Fig. 4A, the NiMn₂O₄/CNT-GCE electrode demonstrated the highest anodic peak current ($I_{pa} = 39.1 \mu\text{A}$) and the lowest peak-to-peak separation ($\Delta E_p = 0.105 \text{ V}$) compared to the NiMn₂O₄-GCE ($I_{pa} = 26 \mu\text{A}$, $\Delta E_p = 0.120 \text{ V}$), CNT-GCE ($I_{pa} = 32 \mu\text{A}$, $\Delta E_p = 0.110 \text{ V}$), and bare GCE ($I_{pa} = 9 \mu\text{A}$, $\Delta E_p = 0.115 \text{ V}$) electrodes, under conditions of 0.1 M KCl with 5 mM [Fe(CN)₆]^{3−}. These findings suggest that the synergistic combination of CNTs and NiMn₂O₄ nanoparticles accelerates electron transfer at the NiMn₂O₄/CNT-GCE interface, facilitating more efficient redox processes.

EIS measurements were conducted to evaluate the interfacial properties of the electrodes, as shown in Fig. 4B. The Nyquist plots, characterized by a semicircle in the high-frequency region representing charge transfer resistance (R_{ct}), revealed distinct differences among the electrodes. The R_{ct} values were determined by fitting the Nyquist plots to a modified Randles equivalent circuit model (Fig. 4B inset), consisting of solution resistance (R_s), charge transfer resistance (R_{ct}), a constant phase element (CPE), and Warburg impedance

(W). Table S1† summarizes the fitted EIS parameters for each electrode. The NiMn₂O₄/CNT-GCE exhibited the lowest R_{ct} value (7.98 Ω), indicating significantly enhanced charge transfer kinetics. In contrast, the NiMn₂O₄-GCE displayed the highest R_{ct} value (316.75 Ω), attributed to the limited intrinsic conductivity of standalone NiMn₂O₄. The bare GCE and CNT-GCE exhibited R_{ct} values of 49.45 Ω and 45.93 Ω , respectively. The slight reduction in R_{ct} for the CNT-GCE reflects the high conductivity of CNTs, which facilitates charge transfer. However, the relatively small improvement suggests that factors such as CNT dispersion and contact resistance on the GCE surface may limit its overall conductivity under the current experimental conditions. The synergy between NiMn₂O₄ and CNTs in the composite is critical to the superior performance of the NiMn₂O₄/CNT-GCE. While CNTs provide a highly conductive framework for electron transport, the integration of NiMn₂O₄ introduces abundant active sites that enhance the electrode's electrocatalytic activity. This balance between conductivity and catalytic efficiency enables the composite to achieve optimal performance, as reflected by its improved CV peak currents and lower ΔE_p values. These findings underscore the complementary roles of CNTs and NiMn₂O₄ in the composite: CNTs contribute to efficient electron transport, while NiMn₂O₄ provides catalytic activity and active sites for redox reactions. Together, these components create a highly effective platform for electrochemical sensing applications.

Additionally, the CV analysis performed at different scan rates (10–100 mV s^{−1}) (Fig. 4C) showed that the redox peak currents (I_{pa}/I_{pc}) increased linearly with the scan rate, confirming that the redox reaction of [Fe(CN)₆]^{3−} at the NiMn₂O₄/CNT-GCE interface was controlled by diffusion. This diffusion-controlled process was further validated by the linear relationship between the peak currents (I_{pa}/I_{pc}) and the square root of the scan rate ($v^{1/2}$), as shown in Fig. 4D. The electrochemically active surface area (EASA) was calculated using the Randles-Sevcik equation as given below:

$$I_p = (2.69 \times 10^5) n^{3/2} A C D^{1/2} v^{1/2}$$

In this equation, I_p represents the redox peak currents, n is the number of electrons involved, v is the scan rate (V s^{−1}), A denotes the electrochemically active surface area (cm²), D is the diffusion coefficient ($7.6 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ for K₃[Fe(CN)₆]), and C is the concentration of [Fe(CN)₆]^{3−} (5 mmol L^{−1}). Based on the slope obtained from Fig. 4D and additional data from Fig. S1† for comparison, the EASA for NiMn₂O₄/CNT-GCE was calculated to be 0.098 cm², which is significantly larger than that of CNT-GCE (0.071 cm²), NiMn₂O₄/GCE (0.048 cm²), and bare GCE (0.064 cm²). The larger EASA of NiMn₂O₄/CNT-GCE can be attributed to the synergistic effects between NiMn₂O₄ nanoparticles and CNTs, which enhance electron transfer and provide a higher density of active sites for electrochemical reactions.²² Combined with its lower resistance and smaller peak separation observed in Fig. 4A and B, these properties

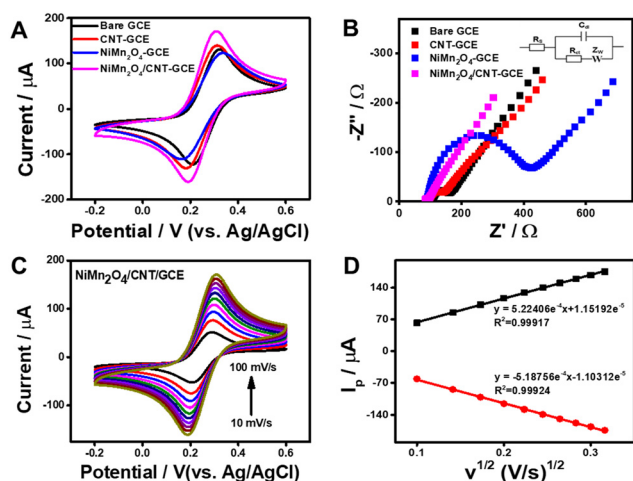
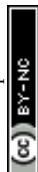


Fig. 4 (A) CV curves of bare GCE, CNT-GCE, NiMn₂O₄-GCE, and NiMn₂O₄/CNT-GCE recorded in 5.0 mM [Fe(CN)₆]^{3−} containing 0.1 M KCl at a scan rate of 50 mV s^{−1}. (B) Corresponding EIS Nyquist plots for bare GCE, CNT-GCE, NiMn₂O₄-GCE, and NiMn₂O₄/CNT-GCE. Inset: Randles equivalent circuit model used for fitting EIS data. (C) CV curves of NiMn₂O₄/CNT-GCE at varying scan rates (10 to 100 mV s^{−1}) in 5.0 mM [Fe(CN)₆]^{3−} containing 0.1 M KCl. (D) Plots of I_{pa} and I_{pc} versus $v^{1/2}$, showing linear relationships.



underscore the remarkable electrochemical sensing potential of NiMn₂O₄/CNT-GCE.

In summary, the integration of NiMn₂O₄ and CNTs onto the GCE significantly enhances its electron transfer kinetics, increases its current response, and expands its active surface area. These synergistic effects establish NiMn₂O₄/CNT-GCE as a promising platform for advanced electrochemical sensing applications.

3.3. Electrochemical behavior of NiMn₂O₄/CNT-GCE for MTX

Fig. 5A illustrates the CV responses of bare GCE, NiMn₂O₄-GCE, CNT-GCE, and NiMn₂O₄/CNT-GCE for the detection of MTX (1 μ M) in 0.1 M PBS (pH 7) at a scan rate of 100 mV s⁻¹. The bare GCE exhibited a low I_{pa} of 22.9 μ A at an E_{pa} of 0.877 V, indicating limited electrochemical activity due to its lack of catalytic properties and active sites. NiMn₂O₄-GCE demonstrated a slight improvement with an I_{pa} of 27.3 μ A and a shift in E_{pa} to 0.846 V, reflecting the catalytic activity and active sites provided by NiMn₂O₄. CNT-GCE exhibited a significant enhancement in electrochemical performance, with an I_{pa} of 120.1 μ A at an E_{pa} of 0.853 V, attributable to the excellent conductivity, high surface area, and efficient electron transport properties of CNTs. Notably, NiMn₂O₄/CNT-GCE showed the highest electrochemical activity, achieving an I_{pa} of 142.6 μ A at an E_{pa} of 0.849 V. This remarkable performance highlights the synergistic effect of CNTs and NiMn₂O₄, where CNTs facilitate electron transfer through their conductive network and NiMn₂O₄ provides abundant active sites for MTX oxidation, resulting in superior sensitivity and electrocatalytic efficiency.

The influence of solution pH on MTX detection was investigated by CV (Fig. 5B and C). As shown in Fig. 5C, the oxidation peak potential (E_{pa}) shifted linearly to lower values with increasing pH in the range of 3–7, with a slope of

−0.0514 V per pH unit ($R^2 = 0.996$). This value is close to the theoretical Nernstian slope of −0.059 V per pH, indicating that the oxidation process involves a proton-coupled electron transfer (PCET) mechanism, likely consisting of one proton and one electron transfer. At pH values above 7, the E_{pa} plateaued, suggesting diminished proton involvement due to reduced protonation of MTX in alkaline media.

To further support this mechanism, the number of electrons (n) involved in the oxidation process was estimated using the Randles–Sevcik equation. The linear dependence of the anodic peak current (I_{pa}) on the square root of the scan rate ($v^{1/2}$) confirms a diffusion-controlled process. The calculated n value was approximately 1, consistent with a single-electron transfer coupled with a proton, as expected in a PCET process. A schematic illustration of the proposed mechanism is shown in Scheme 2, and detailed calculations are provided in the ESI.† The agreement of our findings with previously reported oxidation mechanisms of MTX^{39–41} reinforces the reliability of our proposed pathway. These studies similarly describe a one-electron/one-proton process under comparable pH conditions, supporting the proton-coupled electron transfer mechanism.

Furthermore, the anodic peak current (I_{pa}) reached its maximum at pH 7, highlighting the optimal electrochemical response under neutral conditions. At both lower and higher pH values, the I_{pa} decreased, potentially due to reduced electrochemical activity or electrode surface fouling under extreme acidic or basic environments. These findings emphasize the critical role of pH in modulating the electrochemical behavior of MTX at the NiMn₂O₄/CNT-GCE interface.

The observed pH-dependent electrochemical behavior can be rationalized by the mechanism illustrated in Scheme 2. Initially, MTX undergoes protonation in the solution phase under acidic conditions. This protonation facilitates a

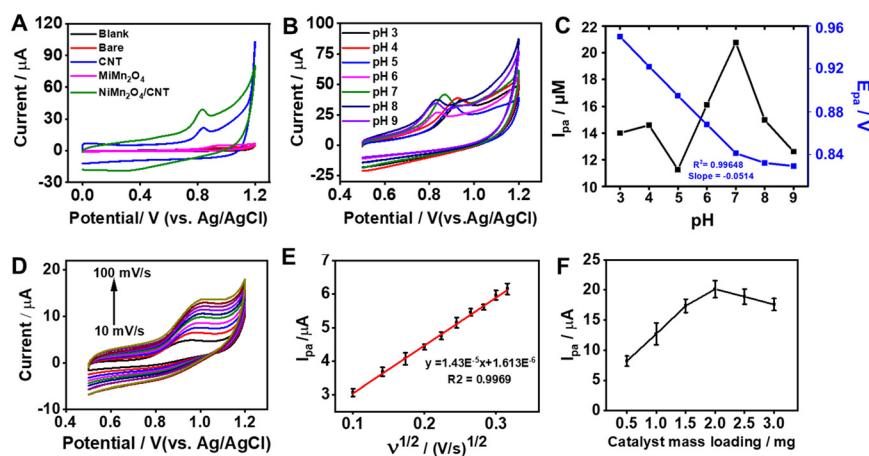
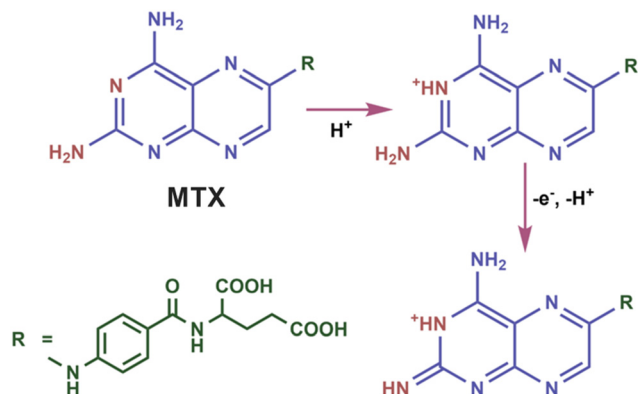


Fig. 5 (A) CV curves of bare GCE, NiMn₂O₄-GCE, CNT-GCE, and NiMn₂O₄/CNT-GCE recorded in 1 μ M MTX at pH 7 with a scan rate of 100 mV s⁻¹. (B) CV responses of NiMn₂O₄/CNT-GCE in 1 μ M MTX under varying pH conditions (pH 3–9). (C) Relationship between pH and I_{pa} (left y-axis) and E_{pa} (right y-axis), showing a slope of −0.0514 V per pH unit ($R^2 = 0.99648$). (D) CV curves of NiMn₂O₄/CNT-GCE at different scan rates (10–100 mV s⁻¹) in 1 μ M MTX. (E) Plot of I_{pa} vs. $v^{1/2}$. (F) Optimization of catalyst mass loading on NiMn₂O₄/CNT-GCE, showing the impact on I_{pa} in 1 μ M MTX.





Scheme 2 Proposed electrochemical oxidation mechanism of MTX at the NiMn₂O₄/CNT-GCE interface.

subsequent stepwise process at the electrode surface, where one electron and one proton are transferred ($-1e^-$, $-1H^+$) to form the oxidized species. The linear relationship between E_{pa} and pH up to pH 7 further supports this proton-coupled electron transfer process. Beyond pH 7, the reduced proton availability likely limits the initial protonation step, leading to the observed saturation behavior.

This proposed mechanism aligns well with experimental data, demonstrating the critical role of PCET in the electrochemical oxidation of MTX. The findings provide valuable insights into optimizing the pH conditions for enhanced electrochemical sensing performance.

The CV response of NiMn₂O₄/CNT-GCE was recorded at varying scan rates ($10\text{--}100\text{ mV s}^{-1}$) in 0.1 M PBS containing 1 μM MTX, as shown in Fig. 5D. The I_{pa} increased linearly with the $\nu^{1/2}$, as presented in Fig. 5E. This linear relationship confirms that the electrochemical reaction is diffusion-controlled, where the redox species near the electrode surface dominate the reaction kinetics. This implies that the availability of MTX molecules at the electrode surface, driven by diffusion, is a critical factor in enabling the PCET mechanism described in Scheme 2. The integration of NiMn₂O₄ and CNTs enhances this process by facilitating efficient electron transfer and providing active sites for the PCET reaction.

The influence of catalyst mass loading on the electrochemical response of NiMn₂O₄/CNT-GCE was investigated using CV, as illustrated in Fig. 5F. The I_{pa} increased proportionally with catalyst mass loading up to 2 mg, reaching a maximum response. Beyond this point, the I_{pa} began to decrease, likely due to increased diffusion resistance and aggregation of catalyst particles on the electrode surface, which hindered efficient electron transfer. These results confirm that 2 mg is the optimal catalyst loading for achieving the best electrochemical performance. By combining this optimized loading with the diffusion-controlled characteristics established in the previous section, NiMn₂O₄/CNT-GCE achieves its peak sensing performance under neutral pH conditions. Based on the experimental findings, the integration of NiMn₂O₄ and CNTs in the

composite plays a dual role. First, CNTs provide a highly conductive network that enhances electron mobility and reduces charge transfer resistance, as evidenced by the EIS results (Fig. 4B). Second, NiMn₂O₄ offers abundant catalytic sites for the PCET mechanism, facilitating efficient redox reactions. Together, these components create a robust platform with the potential for high sensitivity and selectivity in MTX sensing, which will be further validated in subsequent analyses.

3.4. Electrochemical sensing performance of NiMn₂O₄/CNT-GCE for MTX

The electrochemical sensing performance of the NiMn₂O₄/CNT-GCE towards MTX was investigated using DPV in 0.1 M PBS (pH 7.0). Fig. 6A shows the DPV curves obtained at various MTX concentrations ranging from 0.05 to 3.0 μM . A distinct increase in the I_{pa} is observed with increasing MTX concentration, indicating the effective electrocatalytic activity of the NiMn₂O₄/CNT nanocomposite towards MTX oxidation.

The relationship between I_{pa} and MTX concentration is illustrated in Fig. 6B. Notably, the calibration curve exhibits two distinct linear ranges. The first linear range spans from 0.05 μM to approximately 0.1 μM , with a linear regression equation of $y = 1.929e^{-6}x - 6.053e^{-6}$ and a correlation coefficient (R^2) of 0.991. The second linear range extends from approximately 0.1 μM to 3.0 μM , with a linear regression equation of $y = 2.812e^{-6}x + 1.226e^{-5}$ and an R^2 of 0.993. These high R^2 values confirm good linearity within both ranges. This bi-linear response can be attributed to changes in the electrochemical behavior of MTX at varying concentration ranges. At lower concentrations (0.05–0.1 μM), the response is likely dominated by efficient electron transfer kinetics and optimal utilization of available active sites on the electrode surface. As the MTX concentration increases (0.1–3.0 μM), surface saturation effects may begin to play a more significant role, potentially influencing the electron transfer rate and contributing to the change in slope observed in the calibration curve. Despite this change in the dominant mechanism, the diffusion-controlled nature of the MTX oxidation, as evidenced by the results in Fig. 5D and E, ensures consistent and reliable sensing performance across the entire detection range.

The limit of detection (LOD) was calculated using the standard formula,

$$\text{LOD} = 3\sigma/m$$

where σ is the standard deviation of the blank signal, m is the slope of the calibration curve for the lowest concentration range and the LOD, determined using the slope from the first linear range (0.05–0.1 μM), was found to be 0.627 nM.

A comparative analysis (Table 1) highlights the exceptional sensitivity of the NiMn₂O₄/CNT-GCE sensor, demonstrating a lower LOD than previously reported electrochemical MTX sensors. Specifically, it surpasses the



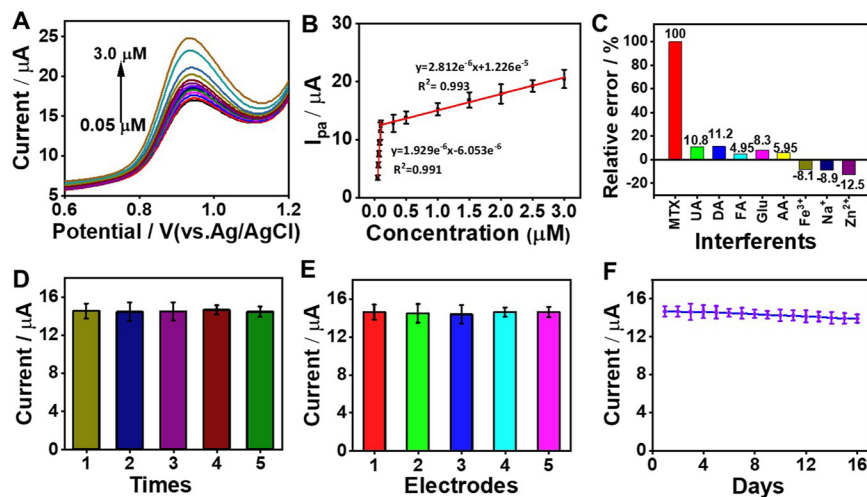


Fig. 6 (A) DPV curves of $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ recorded at various MTX concentrations ranging from 0.05 to 3.0 μM in 0.1 M PBS (pH 7.0). (B) Calibration plot showing the relationship between MTX concentration and I_{pa} . (C) Relative responses of the $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ to various interfering species (50 μM each) added to a 1 μM MTX solution in 0.1 M PBS (pH 7.0), normalized to the MTX response (1 μM MTX without any added interferents, set to 100%). (D) Repeatability of the sensor response to 1 μM MTX, evaluated using five consecutive measurements with a single electrode. (E) Reproducibility of the sensor response to 1 μM MTX, evaluated using five independently fabricated electrodes. (F) Long-term stability of the sensor monitored over 16 days with the electrode stored at 4 $^\circ\text{C}$ in 0.1 M PBS (pH 7.0).

performance of sensors such as $\text{NbO}/\text{NbC}/\text{rGO}/\text{GCE}$ (1.6 nM) and $\text{f-CNT}/\text{PES}$ (2.9 nM). While some sensors, like $\text{Fe}_3\text{O}_4/\text{ppy}/\text{Pd}/\text{SPGE}$ (0.03–100 μM), exhibit broader linear ranges, the linear range of the $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ sensor (0.05–3.0 μM) is well-suited for monitoring clinically relevant MTX concentrations, ensuring practical utility for therapeutic drug monitoring. The combination of a low LOD (0.627 nM) and a relevant linear range positions the $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ sensor as a highly sensitive and practically useful platform for advanced MTX detection.

To evaluate the selectivity of the $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ sensor, the influence of potential interfering species was investigated using DPV. A solution of 1 μM MTX in 0.1 M PBS (pH 7.0) was used as the base solution. Each interfering species (uric acid (UA), dopamine (DA), folic acid (FA), glucose (Glu), ascorbic acid (AA), Fe^{3+} , Na^+ , and Zn^{2+}) was added directly to separate aliquots of this MTX solution to achieve a 50-fold excess concentration (50 μM). The DPV responses of these spiked MTX solutions were then measured as shown in Fig. S2.† Fig. 6C shows the relative responses of the sensor to these species, normalized to the response of the 1 μM MTX solution without any added interferents (set to 100%). Among the tested interferents, Zn^{2+} exhibited the largest relative response (−12.5%), which indicates

a minor level of interference with MTX detection. Zn^{2+} was observed to cause the most significant interference in the electrochemical sensing of MTX, resulting in a current reduction of approximately 12.5%. This interference can be attributed to several factors. First, Zn^{2+} may form weak coordination complexes with MTX, leading to subtle shifts in oxidation potential, which could alter the peak current and affect detection accuracy. Additionally, Zn^{2+} may adsorb onto the electrode surface, influencing charge transfer kinetics and further complicating the sensing process. Finally, competition between Zn^{2+} and MTX for active sites on the electrode surface may reduce the effective binding of MTX, leading to a decreased electrochemical response. These combined effects contribute to the observed interference, underscoring the importance of optimizing sensor selectivity to mitigate such influences. The responses to all other tested species were significantly smaller, further indicating negligible interference. Therefore, the $\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$ sensor demonstrates good selectivity for MTX detection in the presence of common biological metabolites and inorganic ions.

The repeatability, reproducibility, and stability of the fabricated $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ sensor are crucial for ensuring its reliability and practical applicability. The repeatability of

Table 1 A comparative analysis of the linear detection range and LOD for MTX using different methods

Electrode	Technique	Linear range (μM)	LOD (nM)	Ref.
f-CNTPE	SWV	0.01–1.5	2.9	33
$\text{Fe}_3\text{O}_4/\text{ppy}/\text{Pd}/\text{SPGE}$	DPV	0.03–100	7.0	42
MCM-41/ $\text{Fe}_3\text{O}_4/\text{GCE}$	DPV	0.01–50	3	43
3D-graphene CNT/GCE	DPV	0.7–100	70	44
$\text{NbO}/\text{NbC}/\text{rGO}/\text{GCE}$	DPV	0.1–850	1.6	45
$\text{NiMn}_2\text{O}_4/\text{CNT-GCE}$	DPV	0.05–1.0 0.1–3.0	0.627	This work

the sensor was evaluated by performing five consecutive measurements using the same electrode at a fixed MTX concentration of 1 μM in 0.1 M PBS (pH 7.0) (Fig. 6D). The relative standard deviation (RSD) of the peak current for these measurements was calculated to be 0.58%, demonstrating excellent repeatability. Reproducibility was assessed by measuring the response to 1 μM MTX using five independently fabricated $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCEs}$ (Fig. 6E). The RSD of the peak currents obtained from these five electrodes was calculated to be 0.78%, confirming excellent reproducibility of the sensor fabrication method. The long-term stability of the $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ sensor was investigated by storing the modified electrode at 4 $^\circ\text{C}$ in 0.1 M PBS (pH 7.0) and performing measurements daily for 16 days (Fig. 6F). The RSD of the peak current over the 16-day period was calculated to be 1.78%. The sensor retained 94.96% of its initial peak current after 16 days. The slight decrease in current over time may be attributed to gradual degradation of the nanocomposite material or slow leaching of the active material from the electrode surface. These results demonstrate the consistent performance and acceptable long-term stability of the $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ sensor, highlighting its potential for practical applications in MTX detection.

To evaluate the practical applicability of our proposed sensing method for the detection of MTX in real-world conditions, we conducted tests using spiked human serum and urine samples. The performance of the $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ sensor was assessed using the standard addition method. Human serum and urine samples were diluted 10-fold in 0.1 M PBS (pH 7.0) to ensure the MTX concentrations fell within the established calibration range. The resulting solutions were thoroughly mixed before analysis. Fig. 7A and C show the DPV responses obtained in spiked

Table 2 Determination of MTX concentrations in real samples using $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ ($n = 3$)

Sample	Spiked (μM)	Found (μM)	Recovery (%)	RSD (%)
Serum	0.2	0.198	99	3.17
	0.5	0.480	96	2.73
	1	0.980	98	5.26
Urine	0.2	0.196	98	2.35
	0.5	0.490	98	1.30
	1	0.970	97	3.45

serum and urine samples, respectively, at MTX concentrations of 0.2, 0.5, and 1.0 μM . The corresponding calibration plots, illustrating the linear relationship between peak current and MTX concentration, are shown in Fig. 7B and D. The recovery results for both the human serum and urine samples are summarized in Table 2. As shown in Table 2, the recovery rates for MTX in spiked serum samples ranged from 96% to 99%, with RSD values between 2.73% and 5.26% ($n = 3$). In spiked urine samples, the recovery rates ranged from 97% to 98%, with RSD values between 1.30% and 3.45% ($n = 3$). These results demonstrate that the newly developed $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ sensor exhibits adequate recovery rates and acceptable precision for effectively monitoring MTX in complex biological matrices such as serum and urine. The relatively low RSD values indicate good precision and reliability of the proposed method in real sample analysis.

4. Conclusions

A highly sensitive electrochemical sensor for MTX detection was developed by synthesizing a $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite through a process where NiMn_2O_4 nanoparticles, synthesized *via* co-precipitation followed by calcination, were subsequently subjected to hydrothermal treatment in the presence of functionalized multi-walled carbon nanotubes (CNTs). The successful synthesis and synergistic interaction between NiMn_2O_4 and CNTs within the nanocomposite were confirmed through XRD, XPS, SEM, and TEM analyses. The resulting $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ exhibited exceptional electrochemical performance, notably demonstrating an ultralow detection limit of 0.627 nM and a wide linear detection range of 0.05–3.0 μM , significantly surpassing the sensitivity of previously reported MTX electrochemical sensors. This enhanced sensitivity allows for effective monitoring of clinically relevant MTX concentrations. Furthermore, the sensor demonstrated excellent performance in real sample analysis, achieving high recovery rates (96–99%) and low relative standard deviations (RSDs, below 5.26%) in spiked human serum and urine samples, confirming its applicability for accurate and precise MTX detection in complex biological matrices. These combined findings establish the $\text{NiMn}_2\text{O}_4/\text{CNT}$ nanocomposite-based sensor as a highly promising platform for clinical diagnostics and therapeutic drug monitoring of

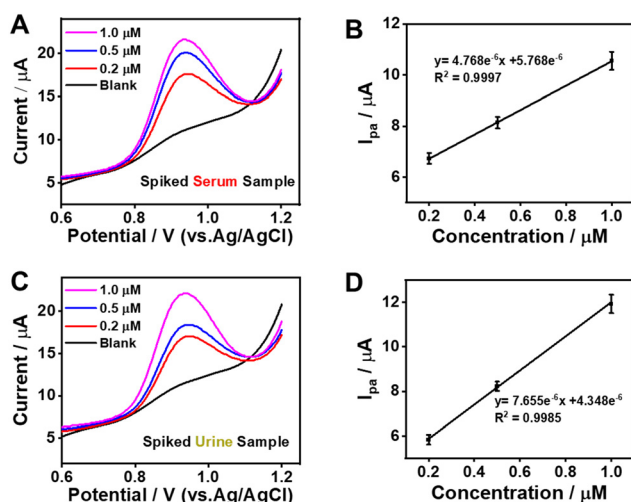


Fig. 7 DPV curves of $\text{NiMn}_2\text{O}_4/\text{CNT}/\text{GCE}$ obtained for (A) spiked human serum and (C) spiked urine samples with increasing MTX concentrations in 0.1 M PBS (pH 7.0). (B) and (D) show the corresponding linear plots between I_{pa} and MTX concentrations for the serum and urine samples, respectively.



MTX. Future research will focus on further enhancing the sensor's selectivity against a wider range of interferents and evaluating its performance in diverse clinical settings.

Data availability

The data supporting the findings of this study are available within the article and its ESI.† No additional datasets were generated or analyzed during the current study.

Conflicts of interest

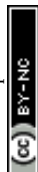
There are no conflicts to declare.

Acknowledgements

This work was financially supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Government (MOE and MSIT) (RS-2021-NR060121 and RS-2025-00559158). This research was supported by Korea Basic Science Institute (National research Facilities and Equipment Center) grant funded by the Ministry of Education (RS-2022-NF000922). This research was supported by 'regional innovation mega project' program through the Korea Innovation Foundation funded by Ministry of Science and ICT (2023-DD-UP-0007). This work was also supported by the Soonchunhyang University research fund.

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