



Single-atom Cu-N/O aerogel nanozymes with dual enzymatic activities for point-of-care detection of multiple neurotransmitters

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ABSTRACT

Single-atom nanozymes (SANs) offer groundbreaking opportunities in enzyme mimicry, yet their broad implementation has been hindered by complex synthesis methods involving extreme conditions. Here, we present a simple, ambient-temperature strategy for fabricating single-atom copper nanozymes embedded in nitrogen/oxygen-functionalized aerogel matrices (Cu-SANs) via spontaneous chelation between copper ions and carbon dots. The resulting Cu-SANs exhibited unprecedented dual enzymatic activities, demonstrating peroxidase- and laccase-like catalytic efficiencies with ~60 and ~400-fold higher, respectively, compared to their natural counterparts. This extraordinary catalytic performance is attributed to the high-density single-atom Cu-N/O coordination sites within the aerogel framework. Leveraging these dual enzymatic capabilities, we developed paper-based microfluidic analytical devices for simultaneous on-site colorimetric detection of acetylcholine and epinephrine. The paper devices exhibited excellent selectivity, sensitivity, stability, and analytical reliability, validated by recovery tests using spiked biological sera samples, including human, rabbit, and bovine sera (>96% recovery, <5% coefficient of variation). This work establishes an efficient, scalable synthetic approach to multifunctional SANs, significantly advancing point-of-care diagnostics for neurotransmitter-related disorders.

1. Introduction

Neurotransmitters are essential molecular messengers that orchestrate neural communication and regulate physiological homeostasis. Among them, acetylcholine (ACh) and epinephrine (Epi) are critically involved in cognitive processing, cardiovascular regulation, and stress response [1]. Dysregulation of these neurotransmitters is a well-recognized hallmark of various neurological and psychiatric disorders, including Alzheimer's disease, Parkinson's disease, depression, and schizophrenia [2,3]. While current analytical techniques — such as high-performance liquid chromatography [4], gas chromatography [5], electrochemical sensing [6], and radioimmunoassay [7] — deliver high sensitivity and accuracy, their clinical implementation remains constrained by expensive instrumentation, complex procedures, and the need for trained personnel [8].

For convenient identification, colorimetric biosensors have garnered significant attention due to their operational simplicity, cost-effectiveness, and visual readout capability [9]. Such attributes make

them highly suitable for point-of-care testing (POCT), enabling rapid diagnostics, decentralized healthcare delivery, and real-time monitoring in resource-limited settings. In this context, natural enzymes have been generally employed, such that horseradish peroxidase (HRP) has served as a cornerstone for detecting various neurotransmitters through inducing direct oxidation or cascade reactions involving H₂O₂ generation, while laccase has been widely utilized for colorimetric detection of phenolic neurotransmitters such as dopamine and Epi [10,11]. However, the intrinsic limitations of natural enzymes — including instability under harsh environments, narrow operating ranges, and high production costs — significantly hinder their widespread practical utilizations.

To address these challenges, enzyme mimics have been intensively studied as robust alternatives to natural enzymes [12]. Among various enzyme mimetics, nanozymes have attracted intense attention due to their exceptional merits including high stability, high and tunable catalytic properties, and cost-effective production [13]. To achieve superior catalytic activity, single-atom nanozymes (SANs) featuring atomically dispersed single metal centers such as Fe, Cu, and Mn have

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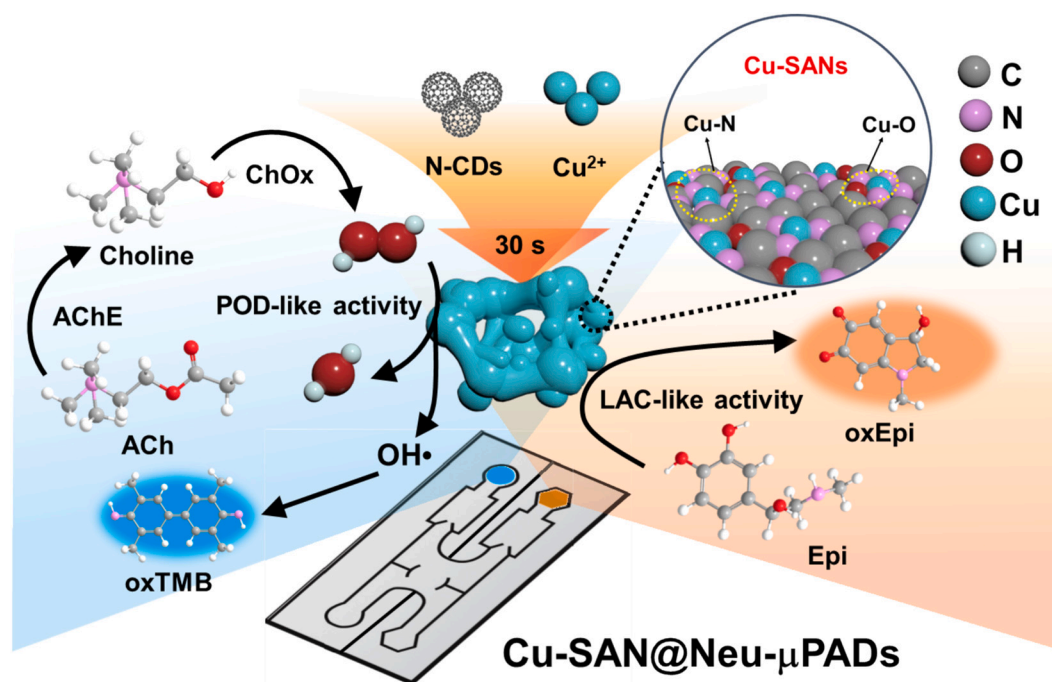


Fig. 1. Schematic representation of the ultrafast synthesis of single-atom copper nanozymes embedded in nitrogen/oxygen-functionalized aerogel matrices (Cu-SANs). The schematic also illustrates the dual enzymatic activities of Cu-SANs (peroxidase- and laccase-like) and the fabrication of Cu-SAN-incorporated paper-based microfluidic analytical devices (Cu-SAN@Neu-μPADs) for simultaneous on-site colorimetric detection of acetylcholine (ACh) and epinephrine (Epi). Abbreviations: N-CDs, nitrogen-rich carbon dots; POD, peroxidase; LAC, laccase.

garnered particular interest due to their revolutionary advantages: maximum atom utilization efficiency, uniform and abundant active sites, exceptional intrinsic activity, and improved catalytic selectivity [14]. These innovations position SANs as next-generation catalytic platforms that can potentially surpass natural enzyme performance while maintaining synthetic advantages [15]. However, the practical utilization of SANs remains severely limited by several critical challenges. First, their synthesis typically requires harsh physicochemical processes including high-temperature pyrolysis, inert atmospheres, and complex multi-step procedures that significantly increase production costs and impede scale-up manufacturing [16]. Second, most reported SANs exhibit mono-functional activities, predominantly mimicking single enzyme types such as peroxidase or oxidase, thereby constraining their application scope in multiplexed detection scenarios where simultaneous analysis of multiple neurotransmitters is essential.

Addressing these limitations, our study presents a breakthrough strategy for developing dual-enzymatic single-atom copper aerogel nanozymes (Cu-SANs) through an ambient-temperature synthesis approach (Fig. 1). We demonstrate that spontaneous chelation between copper ions and nitrogen/oxygen functionalities on carbon dots enables facile formation of three-dimensional aerogel architectures containing atomically dispersed Cu-N/O coordination sites. This synthetic strategy circumvented traditional harsh conditions while achieving exceptional dual enzymatic activities composed of peroxidase and laccase mimicry. Leveraging the dual activities, Cu-SANs were incorporated into paper-based microfluidic analytical devices (μPADs) (Fig. S1), enabling selective, sensitive, precise, and simultaneous quantification of ACh and Epi in POCT environments. This work establishes a new paradigm for scalable, ambient-temperature SAN synthesis while demonstrating exceptional utility for multiplexed neurotransmitter diagnostics in POC settings, potentially revolutionizing decentralized healthcare monitoring for neurological disorders.

2. Experimental section

2.1. Synthesis and characterization of Cu-SANs

Cu-SANs were synthesized via an ion-induced self-assembly using nitrogen-rich carbon dots (N-CDs) as building blocks. N-CDs were prepared from melamine and citric acid as described in the Supplementary Information. Abundant amine and carboxyl groups, derived from melamine and citric acid, respectively, on the N-CDs enabled efficient crosslinking with copper ions during gelation. To synthesize Cu-SANs, 100 μL of copper sulfate pentahydrate (CuSO₄·5H₂O) solution at various concentrations was added to 2 mL of N-CDs solution (2 mg mL⁻¹) and shaken for 30 s at room temperature (RT) to form cyan-blue hydrogels. The hydrogels were collected by centrifugation (10,000 ×g, 10 min), washed three times with deionized water, and lyophilized overnight to obtain Cu-SAN powders.

The resulting Cu-SANs were characterized by scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (EDS), transmission electron microscopy (TEM), high-angle annular dark-field scanning transmission electron microscopy (HAADF-STEM), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), nitrogen physisorption using Brunauer–Emmett–Teller (BET) and Barrett–Joyner–Halenda (BJH) methods, nanoparticle tracking analysis (NTA), and X-ray absorption near-edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) measurements at Cu K-edge. The characterization details were described in the Supplementary Information.

2.2. Evaluation of dual-enzymatic activities of Cu-SANs

Peroxidase- and laccase-like activities of Cu-SANs were evaluated using 3,3',5,5'-tetramethylbenzidine (TMB) oxidation with H₂O₂ and epinephrine oxidation, respectively. For peroxidase-like activity, Cu-SANs (10 μg mL⁻¹) were added to sodium acetate buffer (0.1 M, pH 4) containing H₂O₂ (2 mM) and TMB (1 mM), followed by incubation at 37 °C for 10 min. For laccase-like activity, Cu-SANs (10 μg mL⁻¹) were

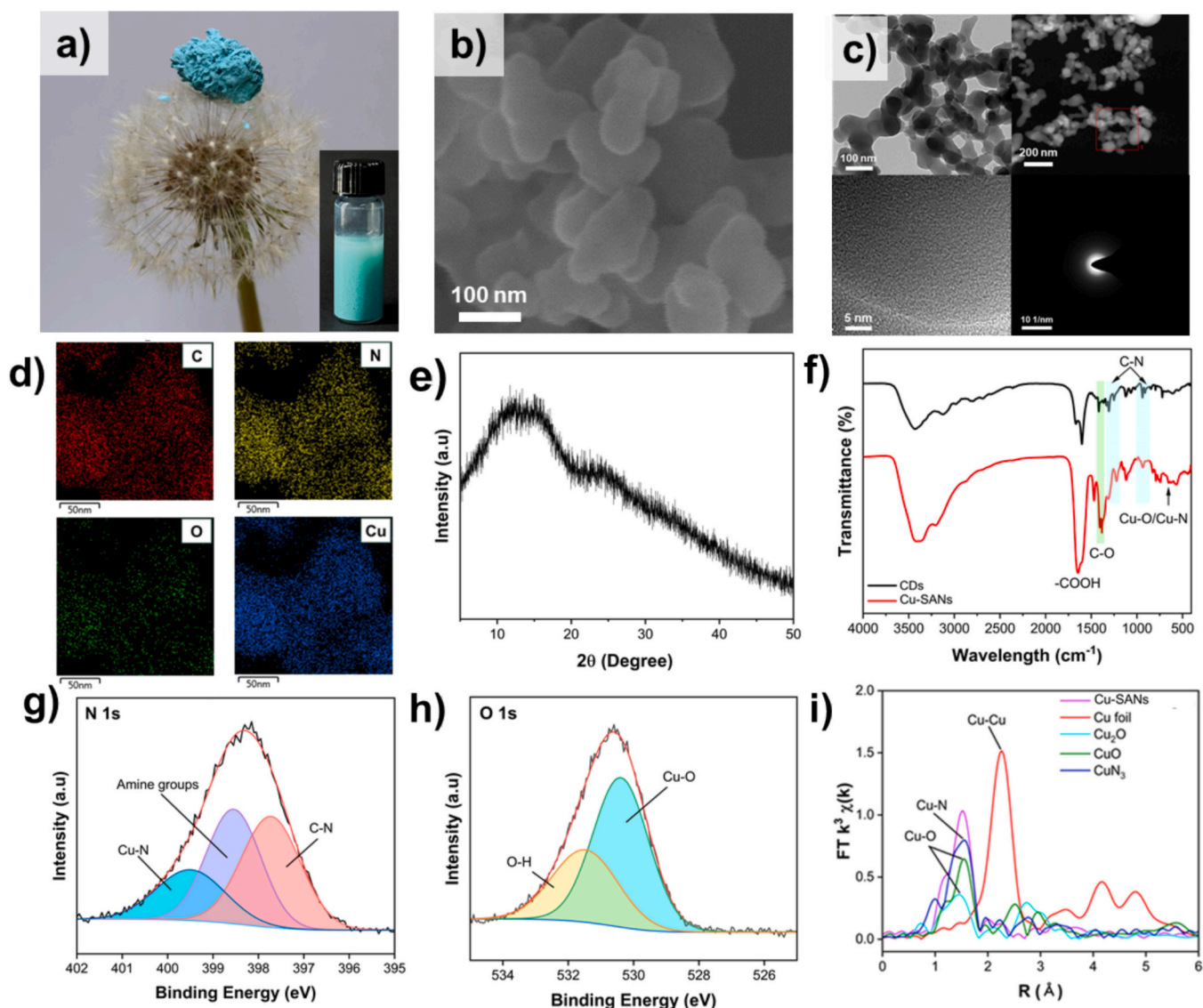


Fig. 2. Structural characterization of Cu-SANs. (a) Photographs of Cu-SANs in powder form and as an aqueous suspension, (b) SEM image, (c) TEM, HR-TEM, and SAED patterns, (d) EDS mapping for C, N, O, and Cu, (e) XRD patterns, (f) FTIR spectra, high-resolution XPS spectra of (g) N 1s and (h) O 1s, (i) Fourier-transformed EXAFS spectra at the Cu K-edge for Cu-SANs and control materials.

added to MES buffer (50 mM, pH 6.8) containing Epi (0.5 mM), and incubated at 37 °C for 10 min. After centrifugation (10,000 ×g, 1 min), absorbance of the supernatant was measured at 652 nm and 485 nm for peroxidase- and laccase-like activities, respectively, using a microplate reader (Synergy H1, BioTek, VT).

The effects of temperature (25–80 °C) and pH (3–10) on both enzymatic activities of Cu-SANs were evaluated following the above procedures. In addition, the effects of oxygen concentration (low, normal, and high) were evaluated for investigating the laccase-like activity of Cu-SANs. Other phenolic substrates such as 2,4-dichlorophenol (2,4-DP), catechol, and hydroquinone (HQ) were also employed rather than Epi for demonstrating the laccase-like activity of Cu-SANs. In this assay, phenolic substrate (0.1 mg mL⁻¹) was mixed with 4-aminoantipyrine (4-AP; 0.1 mg mL⁻¹) in MES buffer (50 mM, pH 6.8), followed by addition of Cu-SANs (10 µg mL⁻¹). The incubation was carried out as described above and the supernatant absorbance at 510 nm was measured. Stabilities of Cu-SANs and their counterpart natural enzymes were also compared in ranges of pH (3–10), temperature (4–120 °C), and long-term storage under static conditions at RT (3 weeks).

Steady-state kinetic assays were performed with varying substrate

concentrations to determine kinetic parameters including Michaelis-Menten constant (K_m), maximum velocity (V_{max}), and catalytic constant (k_{cat}) based on the Michaelis-Menten equation: $v = V_{max} \times [S] / (K_m + [S])$, where v is the initial velocity and $[S]$ is the concentration of substrate. Catalytic constant k_{cat} was derived from $k_{cat} = V_{max} / [E]$, where $[E]$ is the molar concentration of the catalyst [17].

2.3. Colorimetric detection of ACh and Epi based on dual-enzymatic activities of Cu-SANs

Colorimetric detection of ACh was performed using a two-step enzymatic cascade reaction. ACh solutions (50 µL) at various concentrations were mixed with acetylcholinesterase (AChE; 5 U mL⁻¹) and choline oxidase (ChO; 0.5 U mL⁻¹) in PBS buffer (10 mM, pH 7.4, 50 µL), followed by incubation at 37 °C for 10 min. Subsequently, sodium acetate buffer solution (0.1 M, pH 4, 400 µL) including Cu-SANs (10 µg mL⁻¹) and TMB (1 mM) was added and incubated for additional 10 min. The absorbance at 652 nm was recorded using a microplate reader. To assess selectivity, potential interfering molecules at 10-fold higher concentration (1 mM), including phenolic compounds (2,4-DP, HQ),

neurotransmitters (serotonin, Epi), amino acids (phenylalanine), carbohydrates (glucose), and proteins (BSA), were tested with target ACh (0.1 mM). The limit of detection (LOD) was calculated according to the equation: $LOD = 3 S/K$, where S is the standard deviation (SD) of blank absorbance ($n = 5$ measurements) and K is the slope of the calibration plot.

Colorimetric detection of Epi was carried out based on its oxidation by the laccase-like activity of Cu-SANs. Epi solutions (50 μ L) at different concentrations were mixed with Cu-SANs (10 μ g mL⁻¹, 50 μ L) in MES buffer (50 mM, pH 6.8, 400 μ L) and incubated at 37 °C for 10 min. After centrifugation (10,000 $\times$ g, 1 min), the absorbance at 485 nm was measured. Selectivity was demonstrated with interfering molecules at 10-fold higher concentrations (5 mM), including phenolic compounds (2,4-DP, HQ), neurotransmitters (serotonin, ACh, choline), amino acids (phenylalanine), carbohydrates (glucose), and proteins (BSA), compared to target Epi (0.5 mM). The LOD was calculated using the same equation as described above.

2.4. Fabrication of Cu-SAN-incorporated paper-based microfluidic analytical devices (Cu-SAN@Neu- μ PADs) for simultaneously detecting ACh and Epi

Paper-based microfluidic devices incorporating Cu-SANs for multiple neurotransmitters detection (Cu-SAN@Neu- μ PADs) were fabricated using Whatman chromatography paper (Grade 1) by wax printing method. Device patterns were designed using AutoCAD 2018 and printed with a wax printer (ColorQube 8570DN, Xerox). After printing, devices were heated to enable wax penetration and form hydrophobic barriers.

The paper device (22 $\times$ 50 mm) was divided into two sections for ACh and Epi detection, each containing detection/test (T), analyte (A), buffer (B), and control (C) zones (Fig. S1). For ACh detection, Cu-SANs/TMB/sodium acetate mixture was immobilized in detection and control zones, while AChE/ChO/PBS was immobilized in the analyte zone. For Epi detection, Cu-SANs/MES buffer mixture was immobilized in detection and control zones. After sample and buffer addition, the devices were incubated at RT for 10 min. Color changes were observed visually and quantified by analyzing color intensity from smartphone (GALAXY S9 NOTE, Samsung, Korea) images using ImageJ software.

3. Results and discussion

3.1. Synthesis and characterization of Cu-SANs

Cu-SANs were synthesized via a rapid ion-induced self-assembly process using nitrogen-rich carbon dots (N-CDs) derived from melamine and citric acid. Upon mixing N-CDs with copper ions, immediate gelation (<30 s) occurred through chelation between Cu²⁺ and surface functional groups (carboxyl and nitrogen-containing ligands), yielding cyan-blue, ultralight aerogels after lyophilization (Fig. 2a). This rapid coordination-driven gelation effectively immobilizes Cu atoms within the carbon framework, physically restricting their diffusion and aggregation. The simplicity and efficiency of this synthetic approach stand in stark contrast to the complex, energy-intensive pyrolysis methods typically required for SANs, as quantitatively compared in Table S1. SEM and TEM revealed a homogeneous three-dimensional network of hierarchical nanoporous units (diameter of $\sim$ 100 nm) interconnected into an open architecture with abundant air-filled voids (Figs. 2b, c, and S2). EDS mapping confirmed uniform distribution of C (50.79%), N (34.58%), O (4.43%), and Cu (10.20%) (Fig. 2d). BET analysis indicated a surface area of 22.71 m²·g⁻¹ and pore volume of 0.057 cm³·g⁻¹, consistent with typical metallic aerogels [18]. The N₂ physisorption isotherm displayed type II characteristics, confirming the hierarchical porosity of Cu-SANs (Fig. S3).

To gain insights into the architecture of the prepared Cu-SANs, additional characterizations were conducted. High resolution (HR)-

TEM, selected area electron diffraction (SAED), and XRD analyses confirmed the amorphous nature of Cu-SANs, as evidenced by the absence of distinct lattice fringes and crystalline diffraction patterns (Fig. 2c, e). FTIR indicated successful chelation between Cu ions and N-CD surface functionalities, revealing characteristic vibrations of -COOH at 1640 cm⁻¹ [19,20], C-O/C-O-C in a range of 1310–1380 cm⁻¹ [19], and C-N groups at 1250 cm⁻¹ and 938 cm⁻¹ [21,22], alongside subtle peaks associated with Cu-N and Cu-O coordination bonds in a range of 570–750 cm⁻¹ (Fig. 2f) [23–25]. These coordination structures were conclusively verified by XPS analysis. The survey spectrum reveals the appearance of C, N, O, and Cu (Fig. S4a). In the high-resolution peak of Cu 2p, two intense peaks at 932.0 and 951.8 eV represent the Cu 2p_{3/2} and Cu 2p_{1/2} species, respectively. The Cu 2p_{3/2} and Cu 2p_{1/2} were further split into smaller peaks. The signals detected at 931.9 and 951.5 eV attributed to the Cu(I) while the signals at higher energy (934.0 and 953.9 eV) were generated from Cu(II) sites (Fig. S4b) [26], which is presumably due to the partial reduction of Cu(II) to Cu(I) by CDs. The C 1 s peaks at 284.3, 285.5, and 287.0 eV are associated with sp² and sp³ hybridized carbon (C-C/C=C, C-N, and C-O) (Fig. S4c) [27,28]. The deconvolution of N 1 s peak at binding energy 397.7 and 398.6 eV indicate for C-N, and amine ligands, respectively [29–31]. Importantly, the additional peak, centered at 399.5 eV, proves for the presence of Cu-N ligand (Fig. 2g) [32]. Besides, the high-resolution XPS spectrum of O 1 s (Fig. 2h) reveals two peaks at 530.4 and 531.8 eV, respectively, which are assigned to Cu-O and O-H surface species [33–35]. These XPS results strongly support the co-existence of both Cu-N and Cu-O linkages on Cu-SANs.

To clarify the atomic and electronic configuration of Cu species, XANES and EXAFS analyses were performed. The XANES spectra for Cu-SANs were calibrated using Cu foil as a reference (Fig. S4d). The Cu K-edge XANES spectra showed is situated at higher energy than that of Cu foil, indicating the positive charge of Cu species (Cu^{δ+}, 0 < δ) consistent with the XPS results [36]. In the EXAFS spectra, the Fourier-transformed spectra (k³-weighted) in R-space displayed a primary peak at 1.5 Å, attributable to Cu-N/Cu-O coordination (Fig. 2i) [37]. The absence of a Cu-Cu coordination peak at 2.2 Å verified that Cu clusters were not formed in the Cu-SANs [38]. Quantitative fitting of the EXAFS spectra was performed to determine the precise local coordination of Cu atoms. The primary coordination shell is best described by a dominant Cu-N path and a secondary Cu-O path, with the refined structural parameters provided in Fig. S5. The derived coordination numbers correspond to an average local geometry centered on a Cu-N₃ motif, with a fractional oxygen coordination (coordination number of $\sim$ 0.25). This indicates a statistical distribution in which a subset of Cu sites ($\sim$ 25%) incorporates an additional oxygen ligand, forming a Cu-N₃O₁ configuration, while the remaining $\sim$ 75% of Cu sites are coordinated solely by 3–4 nitrogen atoms, forming a Cu-N₃ or Cu-N₄ structure. Unlike high-temperature pyrolyzed SANs with well-defined, symmetric M-N₄ structures, the diversity in the coordination environment around the Cu centers in Cu-SANs is attributable to the aerogel's intrinsic heterogeneity [29,39]. To obtain direct visual evidence of Cu single-atom dispersion, HAADF-STEM imaging was performed (Fig. S6). The images reveal numerous isolated bright dots uniformly distributed across the carbonaceous matrix (indicated by yellow-dash circles), indicating the single Cu atoms. Overall, these results confirmed that Cu-SANs prepared through rapid gelation at RT featured single atomic Cu sites containing plenty of Cu-N/Cu-O sites.

3.2. Dual enzymatic activities and their mechanisms of Cu-SANs

Dual enzymatic activities of Cu-SANs, specifically their peroxidase- and laccase-like activities, were thoroughly examined. The peroxidase-like activity was evaluated through the oxidation of TMB in the presence of H₂O₂. The reaction catalyzed by Cu-SANs yielded a pronounced absorption peak at 652 nm, indicative of oxidized TMB, while control reactions (Cu-SANs + TMB, H₂O₂ + TMB, and TMB alone) showed no

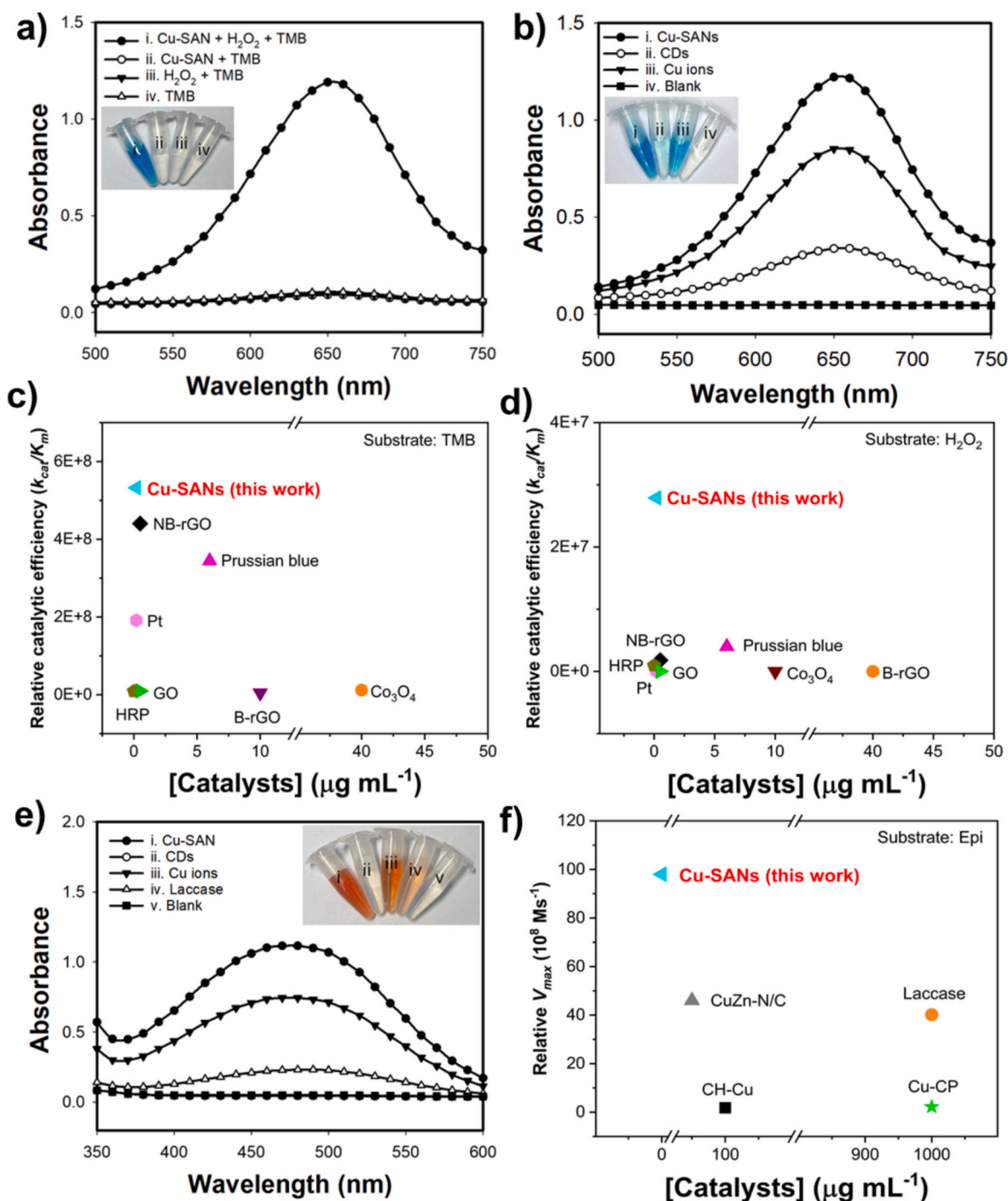


Fig. 3. Evaluation of dual enzymatic activities of Cu-SANs. (a) Absorption spectra of peroxidase-like Cu-SAN-induced color-generating reactions with TMB as the substrate. (b) Comparison of the peroxidase-like activity of Cu-SANs with those of control materials. (c) Steady-state kinetic profiles for peroxidase-like Cu-SANs with TMB as substrate. (d) Steady-state kinetic profiles for peroxidase-like Cu-SANs with H₂O₂ as substrate. (e) Comparison of the laccase-like activity of Cu-SANs with those of control materials. (f) Steady-state kinetic profiles for laccase-like Cu-SANs with Epi as the substrate. Abbreviations: K_m , Michaelis–Menten constant; V_{max} , maximum velocity; k_{cat} , catalytic constant; k_{cat}/K_m , catalytic efficiency.

such absorbance, confirming intrinsic peroxidase-like activity (Fig. 3a). Comparative assays indicated that copper ions primarily drove this catalytic activity rather than N-CDs alone (Fig. 3b). Optimization experiments identified 0.05 M copper sulfate concentration as optimal for gel synthesis, achieving high activity without compromising aerogel morphology (Fig. S7). Temperature and pH optimizations revealed maximal activity at 37 °C and pH 4, establishing these as ideal conditions for subsequent experiments (Fig. S8). The mechanism underlying the peroxidase-like activity was elucidated using radical scavenging assays. Several scavengers, such as superoxide dismutase (SOD,

quencher of $\cdot\text{O}_2^-$) [40], ascorbic acid (AA, quencher of $\cdot\text{OH}$, $\cdot\text{OOH}$, and $\cdot\text{O}_2^-$) [41,42], terephthalic acid (TA, quencher of $\cdot\text{OH}$) [43], sodium azide (SA, quencher $^1\text{O}_2$) [44], isopropyl alcohol (IPA, quencher of $\cdot\text{OH}$) [45], and histidine (His, quencher of $\cdot\text{OH}$ and $^1\text{O}_2$) [46], were added into the peroxidase reactions to detect the presence of intermediate reactive oxygen species (ROS) [47]. The experiments identified hydroxyl radicals ($\cdot\text{OH}$) as the dominant reactive species, with minor contributions from superoxide radicals ($\cdot\text{O}_2^-$) and singlet oxygen ($^1\text{O}_2$) (Fig. S9a). Electron paramagnetic resonance (EPR) spectroscopy using 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin trap provided direct evidence for the

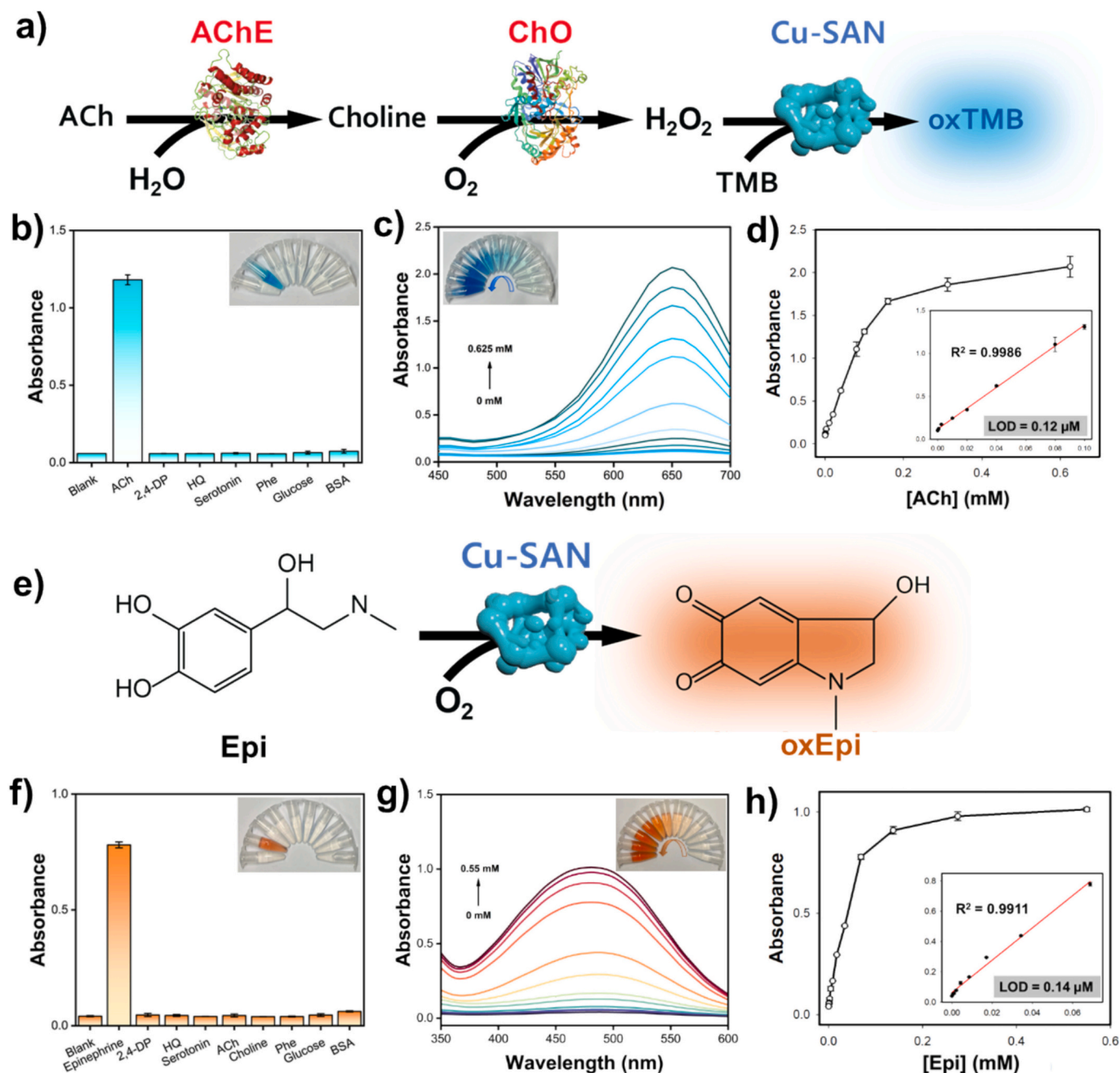


Fig. 4. Colorimetric detection of ACh and Epi in solution. (a) Schematic illustration of ACh detection via a cascade reaction using the AChE/ChO/peroxidase-like Cu-SAN system. (b) Selectivity assay for ACh detection. (c) Absorbance spectra of TMB oxidation at various ACh concentrations and (d) corresponding dose–response curve with linear calibration plots. (e) Schematic illustration of Epi detection based on the laccase-like activity of Cu-SANs. (f) Selectivity assay for Epi detection. (g) Absorbance spectra of Epi oxidation at various concentrations and (h) corresponding dose–response curve with linear calibration plots. All quantitative data represent the mean $\pm$ SD of five independent measurements.

generation of hydroxyl radicals during the peroxidase-like reaction. Upon incubation of Cu-SANs with H_2O_2 , a clear 1:2:2:1 quartet signal corresponding to the DMPO-OH adduct was detected, with its intensity increasing over the 15-min reaction course (Fig. S9b). These findings supported a possible Fenton-type mechanism involving copper-mediated electron transfer and generation of ROS during H_2O_2 decomposition (Fig. S9c) [48,49].

Steady-state kinetic analysis provided detailed insights into the catalytic performance of Cu-SANs (Fig. S10). The apparent K_m values for TMB and H_2O_2 substrates were determined as 0.42 mM and 0.46 mM, respectively, reflecting exceptionally high substrate affinities compared to natural HRP and previously reported nanozymes (Fig. 3c, d, and

Table S2). Correspondingly, high turnover numbers (k_{cat} values of approximately $2.2 \times 10^5 \text{ s}^{-1}$ and $1.3 \times 10^4 \text{ s}^{-1}$, respectively) which are approximately 60- and 4-fold higher than those of HRP ($4.00 \times 10^3 \text{ s}^{-1}$ and $3.48 \times 10^3 \text{ s}^{-1}$), positioned Cu-SANs among the most effective peroxidase mimics currently available (Fig. 3c, d, and Table S2).

Laccase catalyzed the oxidation of phenolic compounds, aromatic amines, and other electron-rich substrates with the concomitant reduction of O_2 to H_2O [50]. Here, the activity was demonstrated by the oxidation of Epi, leading to a visible orange product (adrenochrome) with a characteristic absorbance peak at 485 nm (Fig. 3e). The experiments confirmed copper ions as the active sites responsible for catalytic behavior, rather than N-CDs. Optimal conditions for this reaction were

identified as 37 °C and pH 6.8, mirroring conditions typical for natural laccase activity (Fig. S11). Laccase-like activity was further confirmed by examining the non-production of H₂O₂ during the Cu-SAN-mediated reaction with 2,4-DP, high dependency on dissolved oxygen, and abilities to oxidize other phenolic substrates like 2,4-DP, catechol, and HQ (Fig. S12). The catalytic mechanism was investigated through radical scavenging experiments and EPR analysis, indicating an electron-transfer-driven pathway rather than ROS-mediated oxidation (Fig. S13). Based on these observations, a proposed catalytic mechanism involving direct electron transfer from phenolic substrates to copper active sites and subsequent reduction of molecular oxygen to water was established, consistent with the known mechanism of natural laccase [51,52]. In detail, this process involves three key steps: (1) coordination and deprotonation of the phenolic substrate (e.g., epinephrine) to the Cu (II) center, (2) single-electron transfer from the substrate to Cu(II), generating a phenoxy radical intermediate while reducing Cu(II) to Cu (I), and (3) binding and subsequent four-electron reduction of molecular oxygen to water at the Cu(I) site, regenerating the Cu(II) state.

Steady-state kinetic parameters for laccase-like activity revealed a K_m value of 0.23 mM, which is ~20% lower than that of natural laccase (0.29 mM) (Figs. 3f, S10, and Table S3). Notably, Cu-SANs exhibited a significantly elevated V_{max} value ($\sim 98 \times 10^{-8} \text{ M}\cdot\text{s}^{-1}$) at the same mass concentration, approximately 50-fold greater than natural laccase. In addition, the k_{cat} value was also calculated to be as high as $2.6 \times 10^7 \text{ s}^{-1}$, approximately 400-fold higher than natural laccase, highlighting their exceptional catalytic efficiency (Table S3).

To substantiate that the observed dual enzymatic activities originate from a common pool of copper sites rather than distinct catalytic populations, we performed two control experiments. First, a chelator quenching assay using copper-specific ligands – EDTA, a strong Cu(II) chelator and BCS, a Cu(I)-preferring chelator – was conducted [53,54]. Treatment of Cu-SANs with either chelator resulted in the proportional and simultaneous quenching of both peroxidase-like and laccase-like activities (Fig. S14a). This correlated inhibition confirms that both catalytic functions are mediated by the same copper centers, as sequestration of Cu ions abolishes all activity. Second, to evaluate the versatility of these sites, we tested Cu-SANs in sequential reaction cycles (peroxidase-like followed by laccase-like, and vice versa) with thorough washing between steps. The catalysts exhibited unchanged activity regardless of whether the reactions were performed individually or sequentially (Fig. S14b), demonstrating that the active sites remain fully functional and accessible for both catalytic pathways without cross-inhibition or irreversible modification. These results collectively support the model of versatile single-atom Cu-N/O sites capable of toggling between distinct catalytic mechanisms (radical-mediated vs. direct electron transfer) based on the substrate environment.

The exceptional catalytic performance of Cu-SANs is directly correlated with their unique atomic and electronic structure, as definitively characterized by XPS and XAS. Post-reaction XPS analysis revealed a dynamic redox cycle at the Cu centers: the Cu⁺/Cu²⁺ ratio increased from 1.98 (pristine) to 2.62 after peroxidase-like reactions and to 2.22 after laccase-like reactions. This quantifiable shift confirms the essential participation of Cu⁺ intermediates in both catalytic cycles – acting as the reductant for H₂O₂ activation in the Fenton pathway and as the electron donor for O₂ reduction in the laccase pathway. Concurrently, the O 1 s spectra showed an increase in surface O–H species (from 21.2 at.% to 23.1 at.% and 22.7 at.%, respectively), corroborating the formation of hydroxyl radicals and water as reaction products (Fig. S15a, b). Complementing this, as discussed in Fig. S5, the unsaturated, heterogeneous coordination geometry of Cu-SANs lowers the energy barrier for Cu redox cycling, optimizes substrate binding affinity, and creates an electronically flexible site capable of stabilizing distinct transition states – thereby simultaneously enhancing both radical-mediated peroxidase activity and direct electron-transfer laccase activity. The synergy between this tailored coordination structure and efficient redox cycling constitutes the fundamental origin of the observed dual-enzymatic

enhancement.

Furthermore, stability studies confirmed the remarkable robustness of Cu-SANs, showing consistent catalytic performance under wide-ranging temperatures (4–120 °C), pH conditions (3–10), and prolonged storage durations over 20 days at RT. In contrast, natural HRP and laccase enzymes showed significant activity loss under similar conditions (Fig. S16). On the other hand, Cu-SANs exhibited excellent resistance to metal leaching and structural degradation under operational conditions. This was confirmed by catalyst-filtration and leaching tests, where no further color development was observed after removing Cu-SANs from the reaction mixture, indicating the absence of dissolved Cu species (Fig. S17). ICP-AES analysis further verified that the Cu content remained essentially unchanged before and after catalysis (Fig. S18). In addition, the catalysts preserved more than 90% of their initial activity over five consecutive reaction cycles (Fig. S19), indicating high reusability. Post-reaction characterizations (XPS, XRD, and FT-IR) revealed no formation of Cu nanoparticles or new crystalline phases, and no major changes in functional groups or Cu chemical states (Figs. S15, S20), confirming the preservation of the aerogel framework during catalysis. This exceptional stability highlights the practical advantages of Cu-SANs as multifunctional nanozymes in diverse analytical and biomedical applications.

3.3. Solution-based colorimetric detection of ACh and Epi using Cu-SANs

The dual enzymatic activities of Cu-SANs were employed to develop highly sensitive and selective colorimetric assays for the detection of ACh and Epi in solution. First, a cascade reaction strategy involving AChE, ChO, and Cu-SANs was established for detecting ACh. As depicted in Fig. 4a, ACh is initially hydrolyzed by AChE into choline, subsequently oxidized by ChO to produce hydrogen peroxide (H₂O₂). The generated H₂O₂ is then decomposed by Cu-SANs, leveraging their peroxidase-like activity, resulting in oxidation of the chromogenic substrate TMB, which produces a distinct blue color (oxTMB) measurable at 652 nm. Given that natural enzymes (AChE and ChO) optimally function at neutral pH while Cu-SANs exhibit peak peroxidase activity at acidic conditions (pH 4), a two-step pH strategy was employed. This approach significantly enhanced assay performance compared to single-pH systems, ensuring optimal activity of all involved catalytic units (Fig. S21).

Specificity of the developed assay toward ACh was rigorously evaluated against a variety of potential interfering substances, including phenolic compounds (2,4-DP, HQ), neurotransmitters (serotonin), amino acids (phenylalanine), carbohydrates (glucose), and proteins (BSA). Remarkably, only ACh generated a significant colorimetric signal, even when its concentration was ten-fold lower than interfering substances, confirming the excellent selectivity of the assay (Fig. 4b). Moreover, potential interference from Epi, a neurotransmitter detectable by the laccase-like activity of Cu-SANs, was carefully examined. At the assay's optimal condition (pH 4), minimal interference from Epi was observed, as evidenced by the negligible impact on the characteristic absorption peak at 652 nm, highlighting the assay's ability to discriminate ACh and Epi (Fig. S22). The developed ACh detection system demonstrated exceptional sensitivity, yielding a strong linear correlation between ACh concentration (0.3–100 μM) and absorbance intensity at 652 nm (Fig. 4c, d). The calculated limit of detection (LOD) was 0.12 μM, representing one of the lowest LOD values reported for colorimetric detection of ACh (Table S4).

Epi plays critical roles in physiological processes, including stress response, and its quantitative detection is essential for clinical diagnostics and therapeutic monitoring [55]. Utilizing the intrinsic laccase-like activity of Cu-SANs, a straightforward and indicator-free colorimetric assay for Epi detection was developed. In this assay, Cu-SANs catalyzed the oxidation of Epi directly into adrenochrome, generating a characteristic orange color measurable at 485 nm (Fig. 4e). Control experiments confirmed that no significant color change occurred with potential interfering substances, demonstrating the

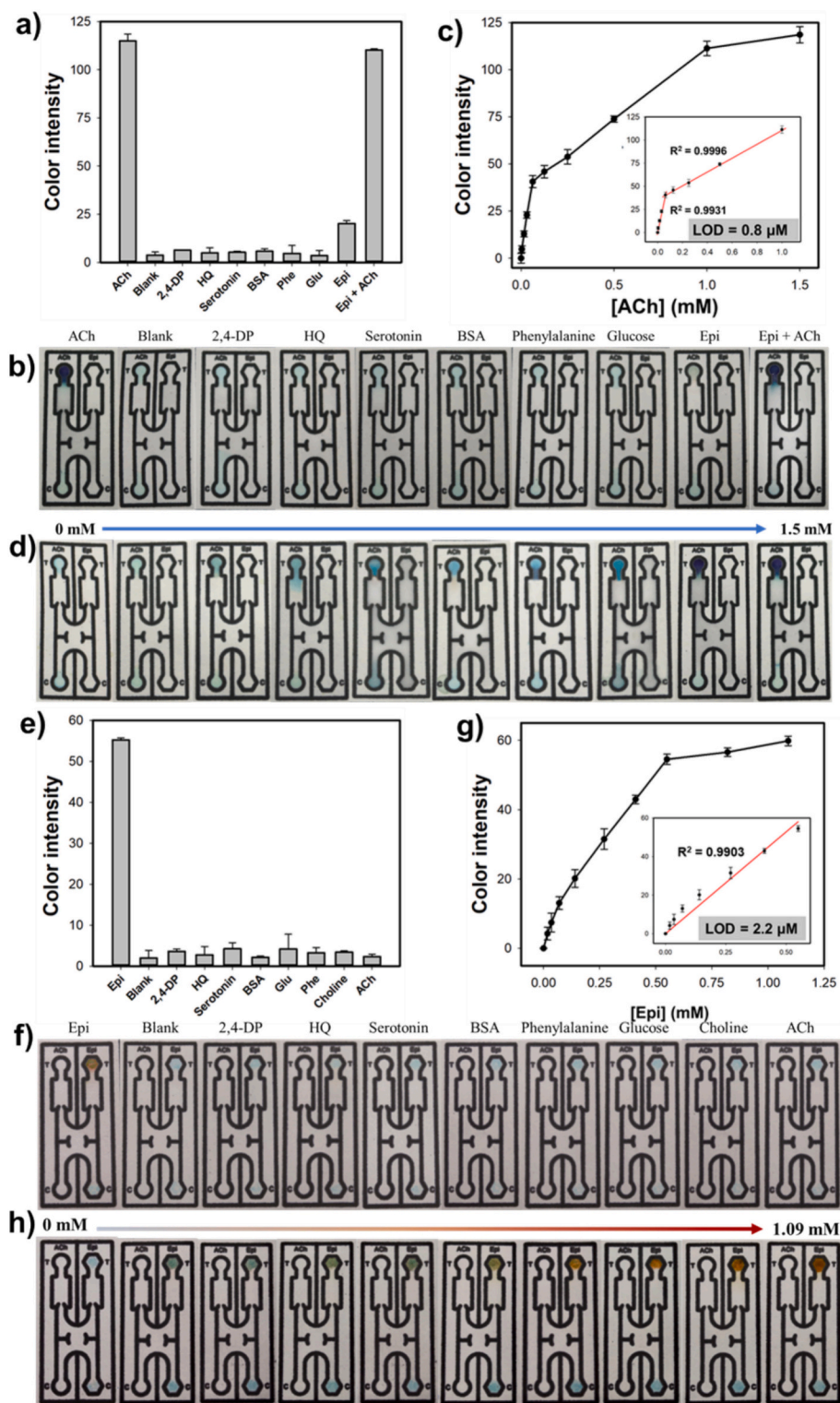


Fig. 5. Analytical performance of Cu-SAN@Neu- μ PADs for on-site detection of ACh and Epi. (a) Selectivity assay for ACh detection with (b) corresponding device images. (c) Dose-response curve and linear calibration plots for ACh detection with (d) corresponding device images. (e) Selectivity assay for Epi detection with (f) corresponding device images. (g) Dose-response curve and linear calibration plots for Epi detection with (h) corresponding device images. All quantitative data represent the mean $\pm$ SD of five independent measurements.

exceptional specificity of the assay (Fig. 4f). Under optimized reaction conditions, increasing Epi concentrations resulted in proportional increases in absorbance intensity at 485 nm and a visually distinguishable color gradient (Fig. 4g). The method exhibited a strong linear response over a broad dynamic range (0.5–70 μM), with a remarkably low LOD of

0.14 μM , among the most sensitive nanozyme-based colorimetric methods for Epi detection reported to date (Fig. 4h, Table S5). All calibration curves for ACh and Epi detection showed excellent linearity ($R^2 > 0.99$) over the tested concentration ranges, with each data point representing the mean $\pm$ SD of five independent measurements ($n = 5$),

Table 1

Accuracy and precision of Cu-SAN-based off-device (solution-based) and on-device (Cu-SAN@Neu-μPAD) assays for quantifying ACh and Epi in spiked human serum samples.

Target	Original ^a (μM)	Added (μM)	Method	Found (μM) ^b	Recovery (%) ^c	SD ^d	CV (%) ^e
ACh	1.3	5	Off-device	6.25	98.4	0.09	1.44
			On-device	6.14	96.7	0.11	1.71
		25	Off-device	26.43	100.3	0.11	0.42
			On-device	26.03	98.8	0.60	2.32
		50	Off-device	50.71	98.8	0.55	1.09
			On-device	50.95	99.2	2.45	4.81
		5	Off-device	4.98	99.6	0.09	1.81
			On-device	4.86	97.2	0.22	4.53
		25	Off-device	24.94	99.8	0.18	0.72
			On-device	24.68	98.7	1.11	4.50
Epi	ND ^f	25	Off-device	51.30	102.6	0.60	1.18
			On-device	49.45	98.9	1.79	3.62

^a The original amount of target in 10-fold diluted human serum.

^b Mean value of five independent measurements.

^c Measured value/expected value × 100.

^d Standard deviation of five independent measurements.

^e Coefficient of variation (CV) = (SD/mean) × 100.

^f Not detected.

demonstrating the high reproducibility of the assay. These results underscore the outstanding analytical performance of Cu-SANs in solution-based neurotransmitter detection.

3.4. Cu-SAN@Neu-μPADs for simultaneous on-site detection of ACh and Epi

To enable convenient detection of neurotransmitters in POCT environments, Cu-SANs were integrated into paper-based microfluidic analytical devices (Cu-SAN@Neu-μPADs), allowing simultaneous on-site colorimetric quantification of ACh and Epi within 10 min (Video S1). The device was designed using AutoCAD software and fabricated by wax-printing onto chromatography paper, as detailed in the experimental section and illustrated in Fig. S1. To ensure device reliability and effectiveness, optimization of key fabrication parameters, including hydrophobic barrier thickness and wax baking duration, was performed. Wax barriers of 0.5 mm thickness combined with a 30-s baking period effectively prevented solution leakage, as confirmed by dye-testing (Figs. S23 and S24). Furthermore, integrated flow-controlling gates significantly minimized undesired backward movement of samples, thereby enhancing the accuracy of the assays (Fig. S25).

For ACh detection, the assay involved a two-step reaction sequence facilitated by distinct pH zones. The analyte zone (A) was buffered at neutral pH (PBS, pH 7.4) to support optimal enzymatic activities of AChE (10 U mL⁻¹) and ChO (1 U mL⁻¹). The detection and control zones were buffered at pH 4 (sodium acetate buffer) to maximize the peroxidase-like activity of Cu-SANs. Subsequently, TMB substrate and Cu-SANs (50 μg mL⁻¹) were immobilized in these zones. Upon introduction of samples and PBS buffer solution, the enzymatic cascade produced H₂O₂, which migrated via capillary action to the detection zone, enabling Cu-SAN-mediated oxidation of TMB to yield a visually distinct blue color. Color intensities were captured using smartphone imaging and quantified through ImageJ software by converting RGB images to grayscale, followed by background correction [29]. The selectivity of this on-device assay was evaluated against various potential interferents including phenolic compounds, neurotransmitters, amino acids, carbohydrates, and proteins. Remarkably, only samples containing ACh showed a significant blue color change, verifying the assay's high selectivity. Although minor color interference was observed in the presence of Epi, the predominant blue signal ensured accurate ACh quantification (Fig. 5a, b). The assay exhibited excellent sensitivity, presenting two linear dynamic ranges from 3.9 to 62.5 μM ($R^2 = 0.9931$) and from 62.5 μM to 1 mM ($R^2 = 0.9996$), achieving a detection limit of 0.8 μM, one of the lowest reported values so far for ACh (Fig. 5c, d,

Table S4).

For Epi detection, MES buffer (50 mM, pH 6.8) was utilized to provide optimal conditions for the laccase-like activity of Cu-SANs. Cu-SANs were immobilized within detection and control zones, and upon addition of Epi samples and MES buffer, Epi was oxidized directly into adrenochrome, resulting in a clear orange color observable by the naked eye after 10 min. Quantitative analysis via ImageJ software further confirmed the excellent selectivity of the assay (Fig. 5e, f). The color intensity increased proportionally with rising Epi concentrations, exhibiting linearity within a dynamic range of 8.7 to 550 μM ($R^2 = 0.9903$) and an impressive limit of detection of 2.2 μM (Fig. 5g, h), which ranks among the lowest reported for sensing Epi to date (Table S5).

To assess practical utility, we evaluated device performance across a range of temperatures and humidities (Fig. S26). The results confirmed that the nanozyme-catalyzed detection of Epi was exceptionally robust, maintaining high accuracy from 4 to 60 °C, which underscores the thermal stability of the Cu-SANs. For ACh, detection remained accurate up to 50 °C; the signal reduction observed at 60 °C is attributed to the denaturation of the natural enzymes (AChE and ChO) in the cascade, not the Cu-SANs. Variations in relative humidity (30–60%) had a negligible impact on the final analytical result for both analytes (Fig. S26c). This comprehensive testing defines a broad operational window for the device and highlights that RT (22 °C) offers the optimal balance of performance for all components, ensuring reliability in common point-of-care settings.

Storage stability and practical utility of the Cu-SAN@Neu-μPAD platform were rigorously assessed. Devices stored at −20 °C maintained near-complete detection performance for one month, while storage at 4 °C resulted in marginal declines, particularly evident for the enzyme-dependent ACh assay (Fig. S27). Clinical applicability was systematically evaluated through recovery and precision analyses in complex biological matrices. Initial validation using spiked human serum samples demonstrated excellent analytical performance, enabling simultaneous quantification of ACh and Epi with recoveries of 96.7–102.6% and coefficients of variation below 5% (Table 1). To further challenge the platform's selectivity and robustness against variations in serum composition, the analysis was extended to rabbit and bovine (calf) sera. As summarized in Table S6, the assays consistently achieved high accuracy across all tested matrices, with recoveries ranging from 97.5% to 102.5%. Moreover, comprehensive precision evaluation—including intra-assay (repeatability) and inter-assay (reproducibility) analyses—confirmed the exceptional reliability of the method, with the coefficient of variation values typically below 5% for both metrics

(Table S7). These results demonstrate that the analytical performance of the platform remains stable and unaffected by diverse biomolecular backgrounds, underscoring its strong resistance to biological interferences and suitability for practical diagnostic applications. Collectively, these findings demonstrate that Cu-SAN@Neu- μ PADs represent a promising portable and sensitive analytical tool for simultaneous neurotransmitter detection in POC and clinical settings.

4. Conclusions

We developed Cu-SANs through an ultrafast gelation process using N-CDs as building blocks, resulting in abundant single-atom Cu-N/O active sites. The synthesized Cu-SANs exhibited exceptional dual enzymatic activities, demonstrating superior peroxidase- and laccase-like performances compared to their natural enzyme counterparts, alongside remarkable stability under harsh conditions. Leveraging these capabilities, highly selective and sensitive colorimetric assays for ACh and Epi detection were established. Furthermore, we developed a simple yet highly efficient Cu-SAN@Neu- μ PADs for simultaneous quantification of these neurotransmitters. This platform showcased excellent selectivity, sensitivity, storage stability, and clinical applicability, achieving detection limits among the lowest reported in the literature. Thus, this work advances practical, robust, and sensitive POC diagnostic platforms, especially for monitoring neurotransmitter-related conditions, with promising implications for future biomedical and clinical applications.

CRediT authorship contribution statement

Thinh Viet Dang: Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Hyun Uk Lee:** Validation, Methodology. **Ji-In Park:** Validation, Methodology. **Jong-Seong Bae:** Validation, Methodology. **Moon Il Kim:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cej.2026.174822>.

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