



Multifunctional cerium nanolabels in electrochemical immunosensing with improved robustness and performance: determination of TIM-1 in colorectal cancer scenarios as a case study

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Abstract

A multifunctional cerium oxide nanoparticles (CeO₂NPs)-based nanolabel is exploited to implement an electrochemical sandwich-type immunoplatfrom for the determination of T-cell immunoglobulin and mucin domain 1 (TIM-1) biomarker, a mucin-like class I membrane glycoprotein associated with cancer angiogenesis. The immunoplatfrom is constructed using screen-printed electrodes where capture antibody is immobilized through the chemistry of diazonium salts. CeO₂NPs exhibit robust pseudo-peroxidase activity even at high substrate concentrations. They are covalently functionalized in a simple manner after carboxylation with a detector antibody (dAb), acting dually as a nanozyme and nanocarrier for sensing bioreceptors. This allows the development of immunoplatfroms with improved robustness and performance (in terms of a moderate enhancement in sensitivity, a significant expansion in the linear range, and a reduction in the background current) compared with the immunoplatfroms prepared using nanolabels also decorated with the natural enzyme (horseradish peroxidase, HRP) or the conventional enzymatic labeling involving the dAb and an HRP-secondary antibody. Under the optimized experimental conditions, the developed electrochemical immunoplatfrom allows the highly sensitive detection of the TIM-1 glycoprotein, with a detection limit of 9.9 pg mL⁻¹ and a linear working range of 33–600 pg mL⁻¹. This performance permits biomarker quantification within clinically relevant ranges. This innovative configuration enables the precise diagnosis and stratification of colorectal cancer patients by analyzing plasma samples without pretreatment beyond a sample dilution and allows establishment of the first cut-off values reported for this purpose.

Keywords CeO₂ multifunctional nanolabels · Nanozymes · Nanocarriers · Electrochemical immunoplatfroms · TIM-1 · Colorectal cancer

Introduction

In recent years, there has been an increasing interest in synthetic systems able to replicate biochemical reactions without relying on the complex structures of natural enzymes. These systems often involve nanomaterials with intrinsic enzyme-like properties and offer significant advantages by mimicking the catalytic behavior of enzymes while overcoming their limitations. By profiting from the robustness and versatility of nanomaterials with enzyme-like activity, these “nanozymes” are poised to have a great impact in fields such as diagnosis, prognosis and monitoring, therapeutics, and in environmental remediation [1–4].

Nanozymes combine inorganic nanomaterials, such as metal or metal oxide nanoparticles, with organic molecules or biomolecules to emulate the catalytic activity of

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natural enzymes [5], showcasing remarkable catalytic properties comparable to biological enzymes. Factors as size, shape, composition, crystal surface, charge, and hydrophilicity influence nanozymes activity levels [6]. In addition, nanozymes exhibit several advantages: (i) high stability; (ii) cost-effectiveness, while enzyme production processes are often complex and costly, inorganic nanomaterials can be produced with high efficiency and at a lower cost; (iii) recyclability, nanozymes maintain their catalytic activity across multiple cycles; (iv) multifunctionality, the huge surface area of nanozymes enables their coupling with multiple ligands fostering multifunctionality. The distinctive characteristics of these materials have propelled significant progress in biosensing, immunoassays, cancer diagnostics and therapy, neuroprotection, stem cell growth, and pollutant removal [7].

Recent advances have revealed amazing enzyme-like characteristics in nanomaterials such as derivatives of fullerenes, gold, rare earths, ferromagnetic nanoparticles [8], and cerium-based materials. These latter have received special attention in the past decade due to their superior redox features, high mechanical strength, good optical properties, thermal stability, and the behavior as nanozymes [9–11]. Indeed, CeO₂ nanoparticles (CeO₂NPs) have been exploited due to their remarkable pseudo-peroxidase activity which is intrinsically linked to their unique crystal structure and redox properties. These NPs adopt a fluorite-type structure, a robust and stable lattice that allows the coexistence of cerium in two oxidation states: Ce³⁺ and Ce⁴⁺. This dual-valence state facilitates the formation of catalytically active redox pairs, enhancing the efficiency of CeO₂NPs as effective mimics of natural enzymes [12]. This behavior is of particular interest for the development of electrochemical biosensors, where their catalytic efficiency can significantly improve biomarker detection and signal generation. Moreover, CeO₂NPs exhibit exceptional versatility for biochemical applications due to their ability to undergo diverse surface modifications. Among these modifications, carboxylation significantly enhances their interaction with biological molecules, such as antibodies. This interaction is achieved through stable covalent bonding with amine groups, which improves the stability of the biomolecules [13]. Taking advantage of the modification ability of CeO₂NPs and their pseudo-peroxidase activity, this work reports their use as multifunctional nanolabels in electrochemical immunosensing. It is important to mention that most of the previous works used these NPs as electrode surface modifiers in label-free strategies [14–16]. Only a few works reported their use as nanolabels in sandwich-type configurations, but always combining CeO₂NPs with other materials (Ag@CeO₂ [17], Co₃O₄@CeO₂-Au@Pt [18]) or even with the natural horseradish peroxidase (HRP) enzyme (CeO₂NPs/MWCNTs-(HRP-Strept)) [19]. These strategies implied more complex preparation and did not take full advantage

of the opportunities CeO₂NPs offer as substitutes for natural enzymes.

Considering this background, this work reports the design, development, and characterization of a competitive electrochemical immunoplatfrom that leverages the multifunctionality of nanolabels formed with CeO₂NPs, functioning as nanozymes and as detection bioreceptors nanocarriers, for the determination of the T-cell immunoglobulin and mucin domain 1 (TIM-1). TIM-1 is a mucin-like class I integral membrane glycoprotein [20] considered as an emerging clinical biomarker associated with aggressiveness in oncological processes and cancer angiogenesis [S1–S5], and other prevalent diseases [S6–S9]. Recent studies have elucidated the capability of TIM-1 to influence the tumor microenvironment. Correlative analyses reveal that its expression levels are in accordance with the presence of aggressive tumors, as heightened expression fosters cell migration and invasion while concurrently inhibits cell apoptosis. Furthermore, recent findings also suggest that the presence of this glycoprotein is associated with enhanced antitumor responses [12], so that the development of targeted simple, low-cost, and robust bioplatfroms able to allow the high detectability and selectivity required for the determination of TIM-1 in real-world clinical scenarios is highly demanded. Due to their unique features, the use of multifunctional CeO₂NP-based nanolabels is considered a good option for implementing robust and affordable electroanalytical immunosensing methods with the required practical performance.

Methods such as enzyme-linked immunosorbent assays (ELISAs) [21], lateral flow immunochromatography [22], and nuclear magnetic resonance (NMR) [23] have been reported for the determination of TIM-1. However, these methods are often time-consuming, expensive, and require complex sample preparation and specialized equipment. Some electrochemical immunosensors for TIM-1 have been already reported [20, 24–27]. However, even though they provide adequate analytical characteristics, they have not been applied in real clinical scenarios. The immunoplatfrom developed and characterized in this work involves the use of screen-printed carbon electrodes (SPCEs) modified by electrografting with *p*-aminobenzoic acid (*p*-ABA) to efficiently immobilize the TIM-1 specific capture biotinylated antibody (btn-cAb) through covalently attached neutravidin (Neu). A sandwich-format immunoassay was thereafter implemented by means of detection antibody (dAb)-decorated CeO₂NPs as multifunctional nanolabels that provide pseudo-peroxidase activity to amplify the electrochemical response. The results obtained demonstrate the role played by these CeO₂NPs-based multifunctional nanolabels in the electrochemical immunosensing of TIM-1 improving moderately or significantly some key characteristics (robustness, sensitivity, linear range, and background current) and

allowing the analysis of samples in a relevant clinical setting (plasma samples from patients with colorectal cancer at different stages).

Experimental

Reagents, solutions, and samples

All commercial solvents and reagents used for the synthesis of dAb/HOOC/CeO₂NPs were used as received, without further purification. Purified water was produced by a Millipore Milli-Q purification system (18.2 MΩ cm). Hydrochloric acid (HCl, 35%) was procured from Merck. Cerium nitrate (Ce(NO₃)₃ × 6H₂O, 99%), ammonium hydroxide (NH₄OH, 35%), sulfuric acid (H₂SO₄, 63–65%), and nitric acid (HNO₃, 65%) were obtained from Sigma-Aldrich. Sodium hydroxide (NaOH) and ethanol (EtOH, 95%) were supplied by Scharlab. 2-(*N*-morpholino)ethanesulfonic acid (MES, ≥ 99.0%) was sourced from GERBU Biotechnik GmbH. Disodium hydrogen phosphate (Na₂HPO₄), sodium chloride (NaCl, ≥ 99.0%), potassium chloride (KCl), and sodium dihydrogen phosphate dihydrate (NaH₂PO₄ × 2H₂O) were acquired from Scharlab.

Additional reagents were of the highest analytical grade available. *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC) was obtained from Across Organics, while *N*-hydroxysulfosuccinimide (sulfo-NHS) was provided by Fluorochem. Hydroquinone (HQ), horseradish peroxidase (HRP), casein, Tween® 20, hydrogen peroxide (H₂O₂, 30% w/v), potassium ferricyanide trihydrate (K₃Fe(CN)₆ × 3H₂O), potassium ferrocyanide trihydrate (K₄Fe(CN)₆ × 3H₂O), HRP-conjugated anti-mouse IgG (RABHRP2), bovine serum albumin (BSA), biotin (btn), and 2,2'-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) were purchased from Sigma-Aldrich. 3,3',5,5'-tetramethylbenzidine (TMB) and neutravidin (Neu) were sourced from Thermo Fisher Scientific. Sodium nitrite (NaNO₂) was obtained from Panreac, and 4-aminobenzoic acid (*p*-ABA) from Across.

All buffer solutions were prepared using purified water. A blocker casein solution (Ref. 37528, casein blocking buffer, BB solution), which was a ready-to-use phosphate buffer solution containing 1% w/v purified casein, and Pierce™ Protein-Free PBS (Ref. 37572, protein-free blocking buffer, PFBB solution) were purchased from Thermo Fisher Scientific. Phosphate-buffered saline (PBS) solutions of 10 mM at pH 7.4, containing 137 mM NaCl and 2.7 mM KCl, 0.05 M phosphate buffer solution at pH 6.0 (PB), 0.025 M MES buffer at pH 5.0, and 0.1 M sodium phosphate buffer composed of PBS with an addition of 0.05% Tween® 20 (PBST) at pH 7.4, were also prepared.

Additional solutions include a mixture of EDC and sulfo-NHS (each at 50 mg mL⁻¹) prepared in 0.025 M MES

buffer at pH 5.0 for the activation of HOOC-CeO₂NPs. For electrochemical measurements, stock solutions of 0.1 M HQ and 0.1 M hydrogen peroxide (H₂O₂) were prepared in 0.05 M PB just before use.

Biotinylated goat anti-human TIM-1 capture antibody (btn-cAb), mouse anti-human TIM-1 detection antibody (dAb), and the recombinant human TIM-1 standard were acquired as the components of the human TIM-1/KIM-1/HAVCR DuoSet ELISA kit purchased from R&D Systems. For the selectivity studies, potential non-target interfering human proteins were evaluated, including hemoglobin (Hb), serum albumin (HSA, ≥ 96%), and serum immunoglobulin G (hIgG), all sourced from Sigma-Aldrich. Additionally, recombinant tumor necrosis factor-alpha (TNF, ≥ 96%), obtained from BD Bioscience and recombinant interleukin-13 receptor alpha-2 (IL-13Rα2, from Human IL-13Rα2 DuoSet ELISA) and recombinant periostin protein (POSTN, from Human Periostin/OSF-2 DuoSet ELISA) procured from R&D Systems, were tested.

Apparatus and electrodes

Amperometric measurements were carried out at room temperature using a CHI812B potentiostat (CH Instruments, Inc.) controlled by the CHI812B software. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) measurements were carried out using a FRA2 μAutolab Type III potentiostat/galvanostat (Metrohm Autolab B.V., The Netherlands) controlled by the GPES & FRA software (Eco Chemie B.V., The Netherlands).

Screen-printed carbon electrodes (SPCEs, DRP-110) with a 4-mm diameter carbon working electrode were sourced from Metrohm-DropSens. These electrodes included a carbon counter electrode and an Ag pseudo-reference electrode. The specific cable connector (DRP-CAC) that provided the interface between the SPCEs and the potentiostat was also obtained from Metrohm-DropSens. The amperometric measurements were taken under constant stirring at 350 rpm using 10 mL glass Pobel electrochemical cells. Additional equipment employed included a Crison Basic 20 + pH meter, a P-Selecta Ultrasons ultrasonic bath, a Heidolph Reax Top homogenizer for small samples, a Meditronic P-Selecta oven, a Velp scientifica magnetic stirrer hot plate, a BA04 Sartorius Secura analytical balance, and an MPW-65R centrifuge from MPW (Med. Instruments).

The synthesized materials were characterized using different techniques with the following equipment: Ultraviolet–visible (UV–Vis) measurements were performed using an HP 8453 UV–Vis diode spectrophotometer controlled by HP Chemstation software; attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was conducted with a Shimadzu FTIR-8300 spectrophotometer and controlled by the OPUS V 5.1 software; transmission

electron microscopy (TEM) images were acquired with a JEM 2100 PLUS microscope operating at 200 kV; and X-ray diffraction (XRD) characterization was carried out using a LynxEye X-ray diffractometer, with Cu K α radiation ($\lambda = 1.54060 \text{ \AA}$), in a Bragg-Bretano configuration.

Preparation of dAb/HOOC-CeO₂NPs

The synthesis of CeO₂NPs was conducted using a precipitation method previously reported in the literature, with minor modifications [S10]. In this procedure, 50 mL of a 0.2 M Ce(NO₃)₃·6H₂O solution was mixed with 25 mL of a 3 M NH₄OH solution under constant stirring at room temperature overnight. A precipitate was formed gradually changing in color from white to gray. The resulting suspension was washed three times with deionized water by centrifugation at 14,000 rpm for 5 min per cycle. The creamy white solid obtained was subsequently dried in an oven at 60 °C for 12 h.

The carboxylation of CeO₂NPs was carried out through a partial oxidation reaction in a (3:1) H₂SO₄/HNO₃ medium following the procedure described by Centane et al. [13]. Specifically, 1 g of the synthesized CeO₂NPs was incubated in 5 mL of the oxidizing mixture with gentle stirring at room temperature for 8 h. The resulting carboxyl-functionalized nanoparticles (HOOC-CeO₂NPs) were washed with a H₂O/EtOH mixture (3:1 ratio) until neutral pH and centrifuged at 14,000 rpm for 5 min. Finally, the nanoparticles were dried in an oven at 60 °C for 24 h.

dAb/HOOC-CeO₂NPs were prepared by using a protocol analogous to that previously reported for the fabrication of nanocarriers involving multi-walled carbon nanotube (MWCNT) nanohybrids [S11, S12]. A 0.2 mg mL⁻¹ aqueous suspension of HOOC-CeO₂NPs was prepared in purified water. The supernatant was removed, and the resulting product was incubated in the dark, under continuous stirring at room temperature for 3 h, with 200 μ L of a solution containing 0.4 M EDC and 0.1 M sulfo-NHS in 10 mM PBS, pH 7.4. The activated HOOC-CeO₂NPs were conjugated by incubation for 24 h at 4 °C under continuous stirring in a 0.5 μ g mL⁻¹ of dAb solution in PBS. The dAb/HOOC-CeO₂NPs nanolabels were centrifuged at 14,000 rpm for 5 min, washed three times with PBS, and finally resuspended in 500 μ L of 100 mM PBST, pH 7.4. The nanocarriers were stored at 4 °C under continuous stirring until use.

Preparation of the immunoplatfom

The functionalization of the SPCEs working electrodes via reductive electrochemical grafting with *p*-ABA was made according to a previously reported protocol [S13]. Initially, diazonium ion (HOOC-Phe-N₂⁺) was synthesized by gradually adding a 2 mM NaNO₂ aqueous solution to a 3 mM

p-ABA solution in 1 M HCl, maintaining the mixture in an ice bath (38 μ L NaNO₂ for 2 mg of *p*-ABA). The reaction was carried out under stirring for 7 min. To covalently bind the diazonium ion to the SPCE surface, each electrode was immersed in the diazonium solution, and ten consecutive voltammetric cycles were applied in the 0.0 to -1.0 V potential range ($\nu = 0.2 \text{ V s}^{-1}$ vs. the Ag pseudo-reference electrode). Finally, the modified SPCEs were thoroughly rinsed with purified water and allowed drying at room temperature.

The carboxyl groups grafted on the working electrode surface (*p*-ABA/SPCE) were activated by depositing 10 μ L of an EDC/sulfo-NHS (100 mM each) solution freshly prepared in 25 mM MES buffer of pH 5.0, allowing the reaction to proceed for 30 min. After washing with the same MES buffer, 5 μ L of a 900 μ g mL⁻¹ Neu solution prepared in MES buffer was added and incubation let to proceed for 45 min. After washing with MES buffer, 5 μ L of a 5 μ g mL⁻¹ btn-cAb solution prepared in 100 mM PBS pH 7.4 was added followed by a 10min incubation time.

After rinsing with PBS, minimization of nonspecific interactions was carried out by blocking with 10 μ L of 1% (w/v) casein allowing incubation for 30 min and followed by a washing step with PBS. The affinity reaction with TIM-1 was accomplished by adding 5 μ L of TIM-1 standard solution (or the samples to analyze) prepared in BB solution onto the working electrode and incubating for 30 min. After washing with PBS, 5 μ L of dAb/HOOC-CeO₂NPs nanolabels was dropped on the modified working electrode surface and incubated for 30 min. The immunosensing platform was rinsed again with 10 mM PBS at pH 7.4 and maintained with a 25 μ L drop of the same buffer until the electrochemical measurements were carried out. All incubation steps during the immunoplatfom assembly were conducted at room temperature in a humid environment to avoid drop evaporation.

Electrochemical measurements

The modified electrode was immersed in a measurement cell containing 10 mL of 50 mM PB at pH 6.0 and 50 μ L of a freshly prepared 100 mM HQ solution. The amperometric measurements were carried out under constant stirring and applying -0.2 V against an Ag pseudo-reference electrode. After the background current was stabilized (~20 s), 50 μ L of a freshly prepared 100 mM H₂O₂ solution in 50 mM PB at pH 6.0 was added [S14]. Due to the pseudo-peroxidase activity of CeO₂NPs, a cathodic reduction current of H₂O₂ mediated by HQ was recorded. Due to the immunoassay format used, the increase in the cathodic current was directly proportional to the TIM-1 concentration. The amperometric responses correspond to the difference between the steady-state current (which was reproducibly recorded in less than 100 s avoiding bias in the results) and the background

current, obtained after and before the addition of H_2O_2 , respectively. In the manuscript, the results are expressed in terms of the Δi values, which correspond to the difference between the amperometric signals measured with immunoplatforms prepared in the presence and in the absence of btn-cAb for each antigen concentration. In addition, unless otherwise specified, they were the mean values of three replicates, and the error bars displayed were estimated as three times the standard deviation of each set of three replicates ($\alpha=0.05$).

The nanolabels and the stepwise fabrication of the immunosensor were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) using a 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution in 0.1 M KCl. In CV, current intensity variations were scanned over a potential range from -0.3 to $+0.7$ V (vs. the Ag pseudo-reference electrode) at a scan rate of 50 mV s^{-1} . EIS measurements were performed under open-circuit conditions, with a sinusoidal excitation amplitude of 10 mV (RMS) applied over a frequency range from 0.04 to 1×10^5 Hz. The automatic analyzer was set to achieve a 0.001% standard deviation (S.D.) for the $I(j\omega)$ correlator output, with a cut-off time of 100 s. Data were collected with 10 points per decade within the specified frequency range.

Evaluation of pseudo-peroxidase activity

To evaluate the pseudo-peroxidase catalytic activity, 1 mg of each compared nanomaterial was placed in a 1.5-mL centrifuge tube. Subsequently, 250 μL of a TMB/ H_2O_2 solution was added, and the development of a blue color was assessed after 2 min of reaction.

To quantify the enzymatic activity (in units per milligram of solid), the standard protocol provided by Sigma-Aldrich was followed [S15]. For this purpose, a 5 mg mL^{-1} ABTS solution was prepared in 0.025 M MES buffer at pH 5.0. The reaction mixture consisted of 40 μL of the suspension of each nanomaterial (1 mg mL^{-1}) and 40 μL of a 0.3% w/w H_2O_2 solution. The absorbance at 405 nm was monitored using a UV–Visible spectrophotometer, with measurements recorded every 20 s over a 3-min period. A blank control was prepared without nanomaterial, following the same procedure.

Analysis of human plasma samples

This study for the biomarker validation was approved by the Ethical Review Board of the Instituto de Salud Carlos III (CEI PI 13_2020-v2). Plasma samples from both healthy individuals (CT) and colorectal cancer (CRC) patients were sourced after the approval of the Ethical Committee from the biobank of Hospital Clínico San Carlos. Written informed consent was obtained from all participants enrolled in the

study. The samples were stored at -80°C until utilized, adhering strictly to ethical guidelines and regulations governing sample handling and experimental protocols.

Following confirmation of the absence of matrix effects in 1/2 diluted plasma samples, the determinations were performed by simple interpolation of the amperometric responses obtained with the immunoplatforms into the calibration plot constructed with external standard solutions. Furthermore, ROC (receiver operating characteristic) curves were constructed using R software (version 3.6.2), and employing the “ModelGood” and “Epi” packages to assess diagnostic performance.

Results and discussion

The immunoplatform developed in this work was constructed on SPCEs modified by *p*-ABA electrografting, followed by covalent attachment of Neu to further immobilize the specific btn-cAb through the affinity reaction between biotin and Neu. After the target capturing, a sandwich assay configuration was implemented by incorporation of the multifunctional dAb/HOOC- CeO_2NPs nanolabels. TIM-1 detection was carried out by monitoring changes in the cathodic current using amperometry in the presence of H_2O_2 and hydroquinone (HQ). This method leveraged the pseudo-peroxidase activity of CeO_2NPs , able to catalyze the H_2O_2 reduction with HQ serving as redox mediator at a potential of -0.2 V (vs. Ag pseudo-reference electrode), producing a cathodic current directly proportional to the concentration of TIM-1. Figure 1 outlines the sequential steps for the preparation of the dAb/HOOC- CeO_2NPs nanocarriers, the construction of the immunoplatform, and the amperometric detection.

Characterization of the nanolabels

The different prepared nanolabels (CeO_2NPs , HOOC- CeO_2NPs , and dAb/HOOC- CeO_2NPs) were exhaustively characterized using morphological, structural, elemental, and electrochemical techniques.

Their shape, size, and spatial distribution were analyzed using TEM (Fig. 2a). Morphologically, unmodified CeO_2NPs exhibited well-defined cubic structures, consistent with that reported in literature [28], while HOOC- CeO_2NPs and dAb/HOOC- CeO_2NPs tended to adopt a spherical shape.

Unmodified CeO_2NPs exhibited higher dispersion compared to HOOC- CeO_2NPs and dAb/HOOC- CeO_2NPs , which showed a more pronounced degree of aggregation. This indicates successful surface functionalization with carboxyl groups and dAb, as hydrogen bonding interactions between these groups likely promote nanoparticle aggregation [29]. The dAb/HOOC- CeO_2NPs displayed a peripheral

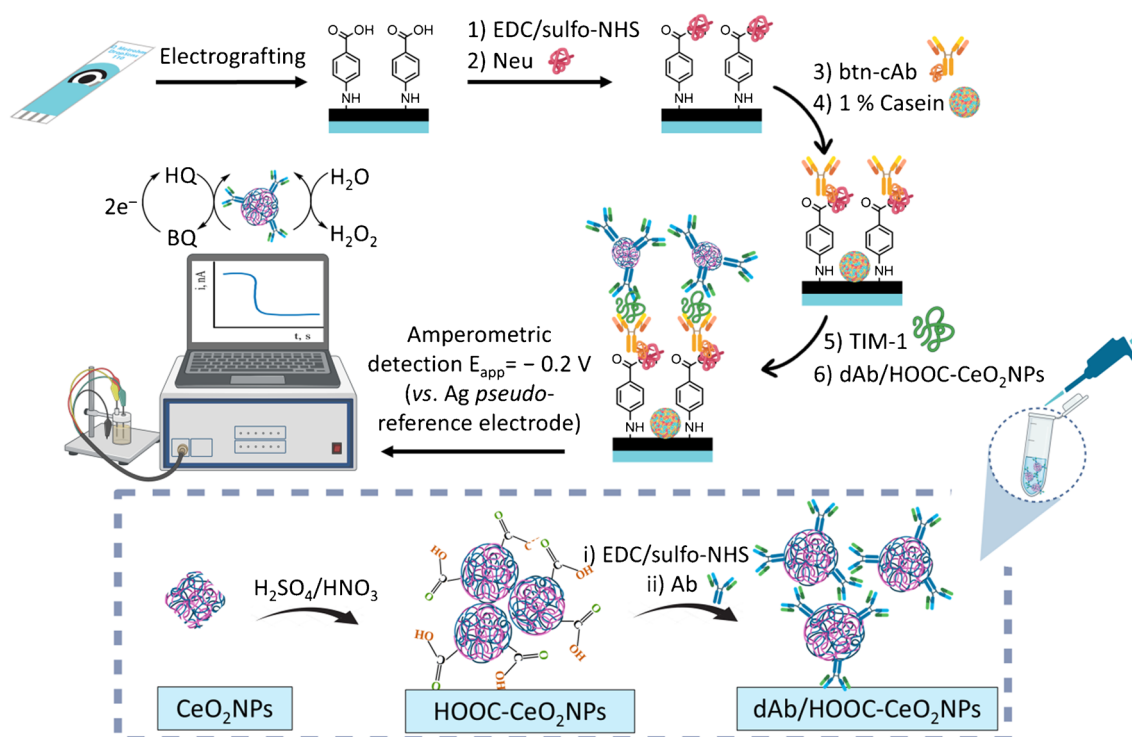


Fig. 1 Schematic illustration of the sequential steps involved in the preparation of dAb/HOOC-CeO₂ NPs nanolabels and the construction of the sandwich immunoplateform for the determination of TIM-1 using amperometric transduction

halo, which should correspond to the presence of the dAb, proving their successful functionalization.

The high resolution of the TEM images allowed for the visualization of lattice fringes, confirming the crystalline nature of the NPs in all cases. Size histograms ($n = 50$) (Fig. 2b) showed a narrow size distribution, reflecting uniform synthesis conditions. The average sizes were found to be (8.9 ± 0.6) nm for CeO₂NPs, (9.6 ± 0.5) nm for HOOC-CeO₂NPs, and (9.8 ± 0.3) nm for dAb/HOOC-CeO₂NPs.

The crystalline structure of the nanomaterials was further characterized using XRD (Fig. 2c). The XRD pattern of untreated CeO₂NPs revealed eight peaks corresponding to the crystal planes (111), (200), (220), (311), (222), (400), (331), and (420), indicative of a fluorite structure [30, 31]. The XRD pattern of HOOC-CeO₂NPs showed similar results, confirming that the crystal structure remained largely unchanged during chemical modification of the NP surface, consistent with the TEM observations and previous studies [13].

The FTIR spectra of CeO₂NPs and HOOC-CeO₂NPs are displayed in Fig. S1a (Supporting Information). For CeO₂NPs, two prominent bands at 1429 cm^{-1} and 1310 cm^{-1} were observed that correspond to the stretching vibration mode of the O-Ce-O bond [S16]. The presence of COOH groups on the surface of HOOC-CeO₂NPs was confirmed by a carbonyl stretching vibration at 1686 cm^{-1} ,

accompanied by additional bands at 1450 , 1282 , 1094 , and 1022 cm^{-1} . The bands at 1282 and 1094 cm^{-1} were associated with the O-Ce-O stretching mode, while those at 1450 and 1022 cm^{-1} were attributed to overlapping Ce-OH vibrations [S17]. Additionally, a band around 2916 cm^{-1} was ascribed to C-H stretching, followed by a broad band corresponding to the -OH groups in the carboxylic moieties [13].

The materials used were further characterized by UV-Vis spectroscopy. As illustrated in Fig. S1b (Supporting Information), CeO₂NPs display an absorption band around 300 nm [S18], corresponding to charge transfers from oxygen 2p to cerium 4f orbitals, confirming the formation of CeO₂ [S19]. The HOOC-CeO₂NPs spectrum is like that of CeO₂NPs, with a slight shift in the absorption bands attributable to resonance effects induced by COOH groups [13].

The electrochemical characterization of the nanolabels was carried out by drop casting modification of the SPCEs working electrode with $5\text{ }\mu\text{L}$ of 1 mg mL^{-1} suspensions of each nanomaterial and allowing drying. Subsequently, CVs and EIS responses were recorded using a 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ in 0.1 M KCl solutions as redox probe (Fig. 3a and b, respectively). The impedance spectra were presented as Nyquist plots and fitted with the appropriate equivalent circuit (Fig. 3c and d).

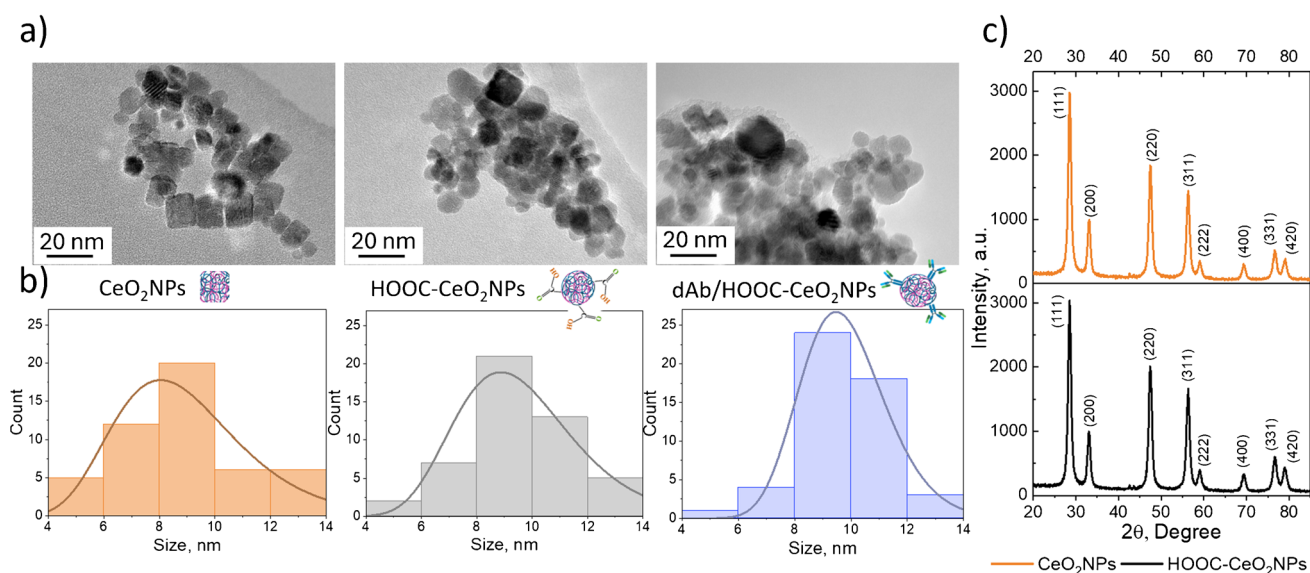


Fig. 2 **a** CeO₂NP, HOOC-CeO₂NPs, and dAb/HOOC-CeO₂NPs TEM micrographs. **b** Histograms of NPs diameter distribution obtained from TEM for $n = 50$ (Gaussian fitting of the particle size distribution

is overlapped in dark line on the histogram bars). **c** Diffraction patterns for CeO₂NPs and HOOC-CeO₂NPs

As shown in Fig. 3a, the CeO₂NPs/SPCE exhibited more reversible behavior and higher current intensity peaks compared to the unmodified electrode (SPCE), indicating that CeO₂NPs enhanced the charge transfer rate. Modification with HOOC-CeO₂NPs resulted in improved current intensities, which was attributed to the enhanced ability of cerium atoms to alternate between Ce³⁺ and Ce⁴⁺ upon carboxylation, generating oxygen vacancies in the fluorite lattice

structure and enhancing electrostatic interactions with anionic electroactive species such as the [Fe(CN)₆]^{3-/4-} probe [12, 32, 33]. These observations were further confirmed by performing voltammograms using Ru³⁺/Ru⁴⁺ as redox probe where an opposite trend was observed upon comparison of CeO₂NPs/SPCE and HOOC-CeO₂NPs (data not shown). The proper immobilization of the dAb on the HOOC-CeO₂NPs was evidenced by a decrease in the anodic and

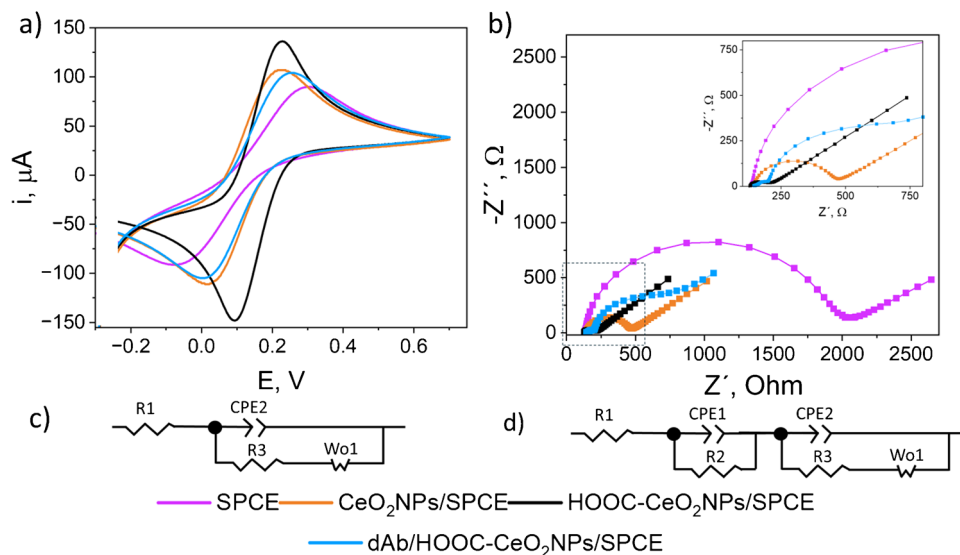


Fig. 3 **a** Cyclic voltammograms (scan rate, 50 mV s⁻¹) and **b** Nyquist spectra (scanning frequencies: 10⁵ – 0.04 Hz; amplitude = 0.01 V, applied potential: 0 V) for the [Fe(CN)₆]^{3-/4-} redox pair. Electrodes: SPCE (purple), CeO₂NPs/SPCE (orange), HOOC-CeO₂NPs/SPCE (black), and dAb/HOOC-CeO₂NPs/SPCE (blue). Equivalent circuits

used to fit the experimental data where R1 is the bulk resistance (R_s), and R2 and R3 are the charge transfer resistances (R_{CT}), CPE is the constant phase element, and W is the Warburg element corresponding to SPCE, CeO₂NPs/SPCE and HOOC-CeO₂NPs/SPCE (c), and dAb/HOOC-CeO₂NPs/SPCE

cathodic peak current intensities reflecting the insulating nature of the antibody and the subsequent decrease of the electron transfer rate at the electrode surface.

Impedance spectra confirmed these observations (Fig. 3b). The charge transfer resistance values (R_{CT}) were obtained by fitting the experimental data to the Randles equivalent circuit illustrated in Fig. 3c (SPCE, CeO₂NPs/SPCE, and HOOC-CeO₂NPs/SPCE) and Fig. 3d (dAb/HOOC-CeO₂NPs/SPCE). As can be seen, the diameter of the semicircle decreased upon modification of the SPCE with CeO₂NPs, indicating a reduction in the R_{CT} attributed to the semiconducting properties of cerium nanoparticles ($R_{CT, SPCE} = 1800 \Omega$; $R_{CT, CeO_2NPs} = 46 \Omega$). Electrodes modified with HOOC-CeO₂NPs displayed an even smaller semicircle diameter, due to the enhanced electrostatic interactions after carboxylation discussed above ($R_{CT, HOOC-CeO_2NPs} = 28 \Omega$). Modification with dAb/HOOC-CeO₂NPs resulted in two semicircles, indicating the coexistence of two charge transfer processes: a smaller semicircle associated with rapid electron transfer influenced by HOOC-CeO₂NPs ($R_{CT 1, dAb/HOOC-CeO_2NPs} = 30 \Omega$) and a larger semicircle suggesting a slower charge transfer due to antibody modification ($R_{CT 2, dAb/HOOC-CeO_2NPs} = 49 \Omega$).

Evaluation of the pseudo-peroxidase catalytic activity and kinetic studies of the nanolabels

CeO₂NPs pseudo-peroxidase activity was evaluated via TMB oxidation in the presence of H₂O₂ [9]. As illustrated in Fig. 4a-I, CeO₂NPs exhibited an absorption band at 300 nm. Upon the addition of 100 μ L of a commercial TMB/H₂O₂ reaction mixture, a new absorption band appeared at 650 nm, corresponding to the blue coloration of the solution (Fig. 4a-II) [S18]. This indicated the formation of the TMB radical cation, demonstrating the pseudo-peroxidase activity of CeO₂NPs. Subsequently, to halt the reaction, 50 μ L of 1 M H₂SO₄ was added, which led to the disappearance of the absorption band at 650 nm and the appearance of a new

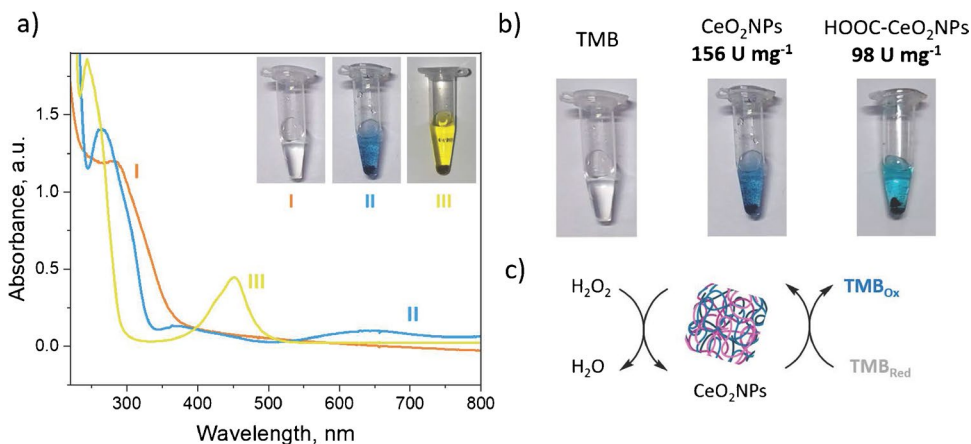
band at 450 nm, observing a solution color change from blue to yellow, which was attributed to the formation of a diimine species (Fig. 4a-III).

The pseudo-enzymatic activity was quantified, in units per milligram of nanozyme/enzyme, using ABTS and following the Sigma-Aldrich protocol equation [S15]. CeO₂NPs pseudo-enzymatic activity was determined to be 156 U mg⁻¹, while that for HOOC-CeO₂NPs resulted to be 98 U mg⁻¹. These results agreed with the color intensity variations observed (Fig. 4b) during the TMB/H₂O₂ reaction (Fig. 4c) and confirmed that the NP pseudo-peroxidase activity increased with surface area [9] and that TMB oxidation was triggered by a Fenton-like reaction induced by CeO₂ and not naturally. Although the synthesized nanozymes exhibited a slightly lower catalytic activity compared to natural HRP (219 U mg⁻¹), this activity was sufficient for developing electrochemical immunoplateforms attractive in terms of reduced cost and improved robustness as it is demonstrated in the following sections.

Amperometric measurements at -0.2 V vs. the pseudo-reference Ag electrode, under constant stirring, were utilized to study the pseudo-peroxidase activity kinetics for CeO₂NPs and HOOC-CeO₂NPs. The variation in the current intensity observed after the addition of increasing concentrations of H₂O₂, ranging from 1.5 to 6.0 mM, into a cell containing 0.5 mM HQ in 50 mM phosphate buffer (PB) at pH 6.0 was used to determine the K_{app} corresponding to the pseudo-peroxidase activity of the synthesized nanozymes.

Firstly, the obtained results were fitted to the Hill equation (Eq. 1), after being linearized according to Eq. 2 where $[S]$ was the substrate concentration (H₂O₂), i was the current intensity variation, i_{max} was the background current intensity at the steady state, and K_M^{app} was the apparent Michaelis–Menten constant. The parameter “ x ,” known as the Hill coefficient, measures the cooperativity of substrate binding to the protein. In this case, when plotting $\log[(i_{max}/i) - 1]$ vs. $\log[H_2O_2]$, a linear relationship was observed with a slope value close to -1 in all cases ($x = (1.1 \pm 0.2)$ for CeO₂NPs, as an example), which reduced Eq. 1 to the Michaelis–Menten

Fig. 4 **a** UV–Vis spectra corresponding to: (I) CeO₂NPs (orange), (II) CeO₂NPs + TMB/H₂O₂ (blue), and (III) after the addition of H₂SO₄ (yellow). **b** Color change observed for TMB/H₂SO₄ upon adding either CeO₂NPs or HOOC-CeO₂NPs. **c** Pseudo-enzymatic cascade reaction mediated by CeO₂NPs or HOOC-CeO₂NPs nanozymes in the presence of H₂O₂, leading to the oxidation of TMB and a consequent color change from colorless to blue



form. This indicated the absence of substrate binding cooperativity, suggesting either a single (or multiple) binding site in the, herein, nanozyme that did not interact cooperatively. This confirmed that the synthesized nanozymes showed a good fit to the Michaelis–Menten model [S20, S21].

$$\frac{i}{i_{\max}} = \frac{[S]^x}{K_M^{\text{app}} + [S]^x} \quad (1)$$

$$\log \left[\left(\frac{i_{\max}}{i} - 1 \right) \right] = \log K_M^{\text{app}} - x \log [S] \quad (2)$$

On the other hand, nanozymes generally exhibit a higher resistance to saturation compared to natural enzymes such as HRP. This distinction arises from the fact that nanozymes function through heterogeneous catalysis, with their activity governed by surface interactions and the availability of active sites. In contrast, HRP can experience saturation at high substrate concentrations due to its reliance on Michaelis–Menten kinetics. Nanozymes, on the other hand, offer a greater density of active sites distributed across their surface. Furthermore, their catalytic performance is not constrained by structural limitations or substrate binding affinities that regulate natural enzymes [S22], as evidenced by the results shown in Fig. S2 (Supporting Information), where the amperometric responses obtained for high H_2O_2 concentrations using HRP-modified SPCEs and dAb/HOOC-CeO₂NPs in the presence of HQ were compared.

Nevertheless, at low substrate concentrations, enzymatic reactions following the Michaelis–Menten model exhibit first-order kinetics. Therefore, by plotting the neperian logarithm of the current intensity over time using the amperometric responses from 1.5 mM substrate solutions (not shown), a regression line was obtained whose slope provided the apparent kinetic constant, K_{app} . The comparison of the K_{app} values calculated for the prepared nanozymes and commercial HRP are provided in Table S1 (Supporting Information).

These results, consistent with those of the spectrophotometric characterization (Fig. 4), showed that CeO₂NPs exhibited the most favorable kinetics among all the NPs tested, although lower than that of the natural enzyme. Moreover, the stepwise functionalization of CeO₂NPs with carboxyl (-COOH) groups and dAb molecules did not significantly impair their pseudo-enzymatic kinetics. All these results suggested the potential of the synthesized nanomaterials as artificial enzymes to develop sensitive and robust immunoplatforms.

Optimization of key experimental variables involved in the immunoplatform preparation and functioning

All experimental variables related to the fabrication and amperometric response of the immunoplatform were

systematically optimized. The criterion for selecting the variables was the maximum ratio obtained from the currents measured in the presence of 500 $\mu\text{g mL}^{-1}$ TIM-1 (T signal) versus those measured in the absence of the target (B signal). The following variables were tested: concentration and incubation time of Neu; concentration and incubation time of btn-cAb; type, concentration, and incubation time of the blocking agent; incubation time for TIM-1; HOOC-CeO₂NPs loading; dAb loading in the dAb/HOOC-CeO₂NPs; incubation time with the dAb/HOOC-CeO₂NPs; and catalytic system used in the electrochemical readout. The results of these optimization studies are provided in Fig. S3 (Supporting Information) and summarized in Table S2 (Supporting Information).

The impact of Neu concentration was evaluated by comparing the amperometric responses of different immunoplatforms prepared utilizing Neu concentrations ranging from 0 to 2000 $\mu\text{g mL}^{-1}$. As shown in Fig. S3a, a larger T/B ratio was achieved for a Neu concentration of 900 $\mu\text{g mL}^{-1}$. Therefore, this concentration was chosen for further work. Regarding the incubation time for Neu binding, a 45-min incubation period was selected, as it provided a better T/B ratio with longer times resulting in a decrease in the specific signal (Fig. S3b) which may be attributed to poorer recognition of TIM-1 since many btn-cAb molecules can be immobilized due to the large Neu loading on the surface. The electrochemical responses of immunoplatforms prepared by varying btn-cAb concentrations (from 0 to 10 $\mu\text{g mL}^{-1}$) (Fig. S3c) showed as high concentrations resulted in lower T/B ratios due to the decrease in the T signals due to impaired target recognition when a lot of btn-cAb was immobilized. Consequently, 5 $\mu\text{g mL}^{-1}$ was selected as the btn-cAb concentration for subsequent experiments. Regarding the btn-cAb incubation time, a 10-min period was found to be sufficient for the affinity interaction between Neu and btn (Fig. S3d). Different blocking agents, commonly used to minimize nonspecific adsorption of immunoreagents on modified electrodes, were tested. As shown in Fig. S3e, a larger T/B ratio was obtained using 2% casein in PBS buffer. Further optimization of casein concentration and incubation time revealed that 1% casein provided better results (Fig. S3f) with a 30-min effective incubation time (Fig. S3g). Larger amounts of casein resulted in a decrease of the specific signals without a significant decrease of the non-specific adsorptions. The target protein incubation time (Fig. S3h) gave rise to a slight increase for the specific signals up to 30 min, then decreasing slightly together with the T/B ratio. Thus, a 30-min incubation period was selected for the effective binding of TIM-1 onto the casein/btn-cAb/Neu/p-ABA-SPCEs. The influence of the HOOC-CeO₂NPs loading was evaluated over the 0 to 1 mg mL^{-1} range. As shown in Fig. S3i, the T/B ratio increased with the HOOC-CeO₂NPs loading, reaching

a higher value for 0.2 mg mL^{-1} . Larger loadings provided leveled off non-specific signals while T decreased probably due to nanoparticle aggregation preventing binding of the dAb. The effect of the dAb solution concentration used to decorate the $\text{HOOC-CeO}_2\text{NPs}$ yielded a larger T/B ratio for $0.5 \text{ } \mu\text{g mL}^{-1}$, which was therefore selected (Fig. S3j). The incubation time for the dAb/ $\text{HOOC-CeO}_2\text{NPs}$ bioconjugate was checked over the 15 to 90 min range. As shown in Fig. S3k, the intensity of specific signals decreased after 30 min, likely due to a more impeded recognition of the target or to possible aggregation phenomena. Furthermore, since we were working with a pseudo-enzyme, the effect of the substrate and redox mediator concentrations and the potential to be applied for amperometric detection were evaluated. The effect of H_2O_2 concentration was checked over the 0.25 to 5 mM range, with the results depicted in Fig. S3l. As expected, the specific current increased rapidly with the H_2O_2 concentration, with the dAb/ $\text{HOOC-CeO}_2\text{NPs}$ showing an exceptional stability even at high H_2O_2 concentrations. This remarkable stability was attributed to the ability of CeO_2NPs to toggle between Ce^{3+} and Ce^{4+} oxidation states, thus endowing them with a notable advantage in terms of regenerating their active state, thereby potentially enhancing their catalytic efficiency in contrast to the natural HRP which is prone to denaturation for high H_2O_2 concentrations (as shown by the results in Fig. S2) [S23]. These results highlighted the enhanced robustness of synthetic nanozymes compared to their natural enzyme counterparts. Considering the T/B ratios, the selected H_2O_2 concentration was 1 mM. Moreover, Fig. S3m shows as the T/B ratio increased with the HQ concentration up to 1 mM, then decreasing for larger concentrations which is likely due to electrode fouling.

Figure S4a shows the CV behavior of the HQ system in the presence of H_2O_2 , at the immunosensor. When compared with the voltammogram obtained at the bare SPCE, a splitting of peaks was observed. This can be attributed to a mechanistic change caused by the interaction between HQ and the antibody, as reported in the literature [34]. The second reduction peak appeared at around -0.15 V , and therefore a slightly more negative potential was necessary to fully reduce the electroactive species. At potential values close to -0.3 V , an increase in the faradaic currents was seen due to the background reduction. This behavior was corroborated by performing amperometric measurements at different potentials (Fig. S4b), showing that the background current in the absence of TIM-1 increased for potential values more negative than -0.3 V . Although a larger T/B ratio was observed at -0.3 V , considering the minimal differences observed between -0.2 and -0.3 V , and as a compromise between sensitivity and selectivity, -0.2 V was selected as the potential to be applied for amperometric detection.

Stepwise electrochemical characterization of the immunoplateform preparation

The immunoplateform preparation, using the experimental conditions selected above, was monitored step by step by CV and EIS, using $5 \text{ mM Fe(CN)}_6^{4-/3-}$ as redox probe. The corresponding Nyquist plots are shown in Fig. S5a and S5b (Supporting Information). As observed, the modification of the SPCE with *p*-ABA provoked a significant increase of the charge transfer resistance ($R_{\text{CT, SPCE}} 1800 \text{ } \Omega$; $R_{\text{CT, } p\text{-ABA/SPCE}} 11000 \text{ } \Omega$), which was attributed to the electrostatic repulsion between the anionic redox probe and the $-\text{COO}^-$ groups present on the electrode surface under the measurement pH conditions (Fig. S5a). The subsequent activation with EDC/sulfo-NHS led to a reduction in the R_{CT} value due to the neutralization of the $-\text{COO}^-$ groups ($R_{\text{CT EDC/sulfo-NHS/} p\text{-ABA/SPCE}} 5200 \text{ } \Omega$). The R_{CT} value increased again after the immobilization of Neu ($R_{\text{CT, Neu/} p\text{-ABA/SPCE}} 15000 \text{ } \Omega$), due to the formation of a protein layer that inhibited charge transfer on the electrode surface. The subsequent immobilization of the btn-cAb resulted in a decrease of the electron transfer resistance $R_{\text{CT, btn-cAb/Neu/} p\text{-ABA/SPCE}} 10000 \text{ } \Omega$), possibly due to a reorganization of the Neu layer induced by its interaction with the btn-cAb [S24]. Incubation with 1% casein led to a drastic decrease in R_{CT} (Fig. S5b). It is important to mention that, from this step, the impedance spectra showed two different semicircles corresponding to an electrode surface on which two layers are deposited during the immunoplateform preparation. A similar behavior was reported previously for other immunosensors involving incubation with casein [S11, S25]. The equivalent circuit $R_1(R_2Q_1)(Q_2[R_3W_1])$ displayed in Fig. S5d corresponded to an electrode surface coated with two layers and best fitted the experimental results ($R_{\text{CT, casein/btn-cAb/Neu/} p\text{-ABA/SPCE}} 490 \text{ } \Omega + 2600 \text{ } \Omega$). The incorporation of the target protein TIM-1 caused an increase in the second semicircle area in the Nyquist plot, consistent with the formation of a layer that made charge transfer more difficult ($R_{\text{CT, TIM-1/casein/btn-cAb/Neu/} p\text{-ABA/SPCE}} 420 \text{ } \Omega + 2800 \text{ } \Omega$). Finally, the addition of the nanocarriers (dAb/ $\text{HOOC-CeO}_2\text{NPs}$) resulted in a decrease in R_{CT} ($R_{\text{CT, dAb/HOOC-CeO}_2\text{NPs/TIM-1/casein/btn-cAb/Neu/} p\text{-ABA/SPCE}} 290 \text{ } \Omega + 2500 \text{ } \Omega$), attributed to the conductive nature of CeO_2NPs , which facilitated electron transfer. The observed behavior aligned with the results observed by CV (Fig. S5c and S5d).

Analytical operational characteristics and selectivity

The analytical and operational characteristics of the immunoplateform developed for the determination of TIM-1 were evaluated. To check the advantages of the used nanolabel, the calibration plots constructed with immunoplateforms prepared using nanolabels decorated with and without HRP

(dAb + HRP/HOOC-CeO₂NPs and dAb/HOOC-CeO₂NPs) as well as using the conventional enzymatic labeling involving the dAb and an HRP-secondary antibody (HRP-anti-mIgG) were compared. The respective calibration graphs are displayed in Fig. 5. The synthesized HOOC-CeO₂NPs nanocarriers without HRP improved the sensitivity by a 12% and widened the linear concentration range compared to that provided using dAb + HRP/HOOC-CeO₂NPs. The use of dAb/HOOC-CeO₂NPs (black line in Fig. 5) allowed a strong linear correlation between the amperometric response and TIM-1 concentration in the 33–600 pg mL⁻¹ concentration range. The corresponding fitting equation was $-i$ (nA) = (2.38 ± 0.06) [TIM-1, pg mL⁻¹] + (98 ± 22), with an excellent correlation coefficient of $r^2 = 0.995$. However, when the dAb + HRP/HOOC-CeO₂NPs nanomaterial was employed (red line), a shorter linear concentration range, up to 200 pg mL⁻¹, and a smaller slope value were apparent, $-i$ (nA) = (2.1 ± 0.2) [TIM-1, pg mL⁻¹] + (475 ± 16), with a correlation coefficient of $r^2 = 0.990$. These results showed clearly that the dAb/HOOC-CeO₂NPs nanolabel provided sufficient pseudo-peroxidase activity and did not require decoration with HRP to provide immunosensing with the required sensitivity. It is worth noting also the significantly higher intercept value and the shorter linear range obtained using dAb + HRP/HOOC-CeO₂NPs nanolabels. The latter can be attributed to the lower number of dAb molecules on the NPs when HRP molecules were also incorporated.

The comparison with the conventional strategy using recognition and labeling with HRP-anti-mIgG/dAb also showed a slightly higher sensitivity ((2.38 ± 0.06) vs. (2.0 ± 0.1) nA mL pg⁻¹), a broader linear range, and an intercept almost 10 times lower (98 vs 978 nA) using the multifunctional

nanolabel (dAb/HOOC-CeO₂NPs), highlighting the advantages it offers in electrochemical immunosensing.

The limit of detection (LOD = 3 × s_B /slope) and limit of quantification (LOQ = 10 × s_B /slope) values for the dAb/HOOC-CeO₂NPs-based immunoplatfrom were estimated to be 9.9 and 33 pg mL⁻¹, respectively (s_B , standard deviation of the blank, $n = 10$).

A study on TIM-1 levels in plasma from patients with clear-cell carcinoma established a threshold of 142 pg mL⁻¹ [35]. Therefore, it can be concluded that the LOD and LOQ values achieved with the developed immunoplatfrom allow the clinical differentiation between healthy individuals and cancer patients.

Table 1 compares the analytical performance and main characteristics of the developed immunoplatfrom with those reported with other electrochemical immunoassays and immunosensors for TIM-1. In terms of analytical performance, the LOD achieved was comparable to some reported values [20], although higher than others [24, 26], but still within the clinically relevant range mentioned above. It is also important to highlight here that none of the reported electroanalytical methods for the determination of TIM-1 were applied in real scenarios.

Interestingly, the developed immunoplatfrom also exhibited a slightly better LOD compared to the commercial ELISA kit using the same immunoreagents (9.9 pg mL⁻¹ vs. 15.6 pg mL⁻¹ ELISA Kit). Additionally, it avoided the need for expensive enzymes, offered a significantly faster detection time (60 min vs. 4 h 30 min starting from the casein/btn-cAb/Neu/p-ABA-SPCEs and cAb-coated ELISA plate, respectively), and can be used with portable, cost-effective instrumentation, making it ideal for decentralized settings and in field applications.

Fig. 5 **a** Calibration graphs for the determination of TIM-1 constructed with immunoplatforms involving the use of dAb/HOOC-CeO₂NPs (black line), dAb + HRP/HOOC-CeO₂NPs (red graph), and HRP-anti-mIgG/dAb (blue graph) for the recognition and enzymatic labeling of the target TIM-1. **b** Amperometric signals recorded with the dAb/HOOC-CeO₂NPs-based immunoplatfrom for different TIM-1 concentrations

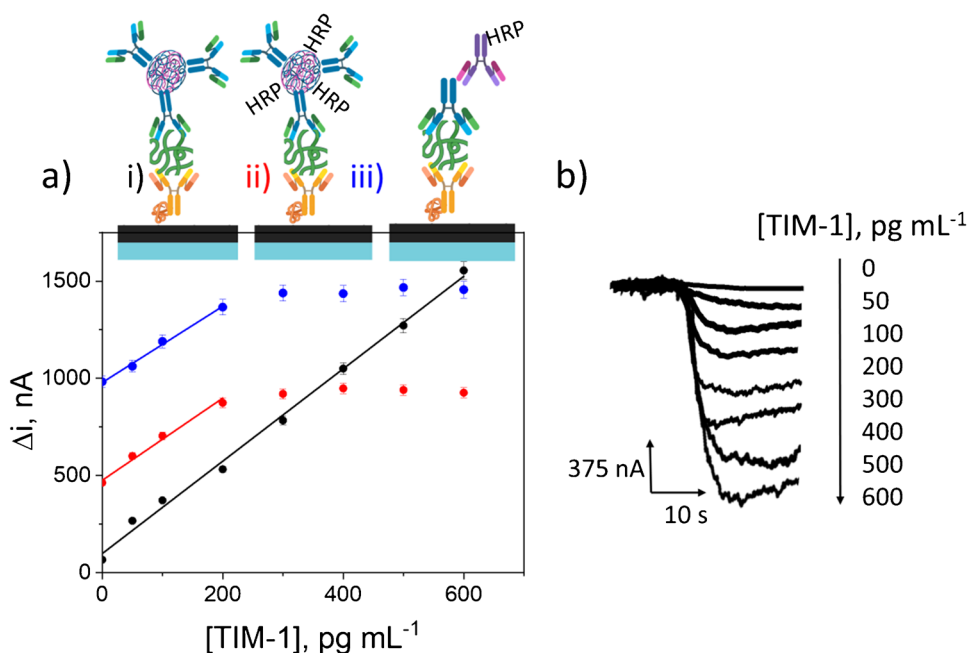


Table 1 Analytical performance and key features of electrochemical immunoassays/immunosensors reported for the determination of TIM-1

Detection strategy	Technique	Linear range, pg mL ⁻¹ /LOD, pg mL ⁻¹	Assay preparation time/test time	Storage stability	Sample	Reference
Sandwich immunoassay with cAb on AuNPs-modified GCE, and dAb-decorated ABEL-PEI-PFO dots-RGOs/PtNPs as nanolabels	ECL	0.0500–1000/0.0167	cAb/AuNPs/GCE ~ 13 h; ABEL-PEI-PFO dots-RGOs/Pt NP@Ab ₂ -BSA ^o ; ~ 18 h/not reported	Not reported	Spiked serum	[24]
Sandwich immunoassay with specific cAb on CuCl ₂ NWs and AuNP-modified GCE, and dAb-decorated PdPiBP MNPs/MXene as nanolabels	DPV	500–100000/86	cAbs/CuCl ₂ NWs/AuNPs/GCE ~ 2 h; dAb/PdPiBP MNPs/MXene: ~ 49 h; CuCl ₂ NWs: ~ 10 min/ ~ 2 h	cAbs/CuCl ₂ NWs/AuNPs/GCE, and PdPiBP MNPs/MXene nanolabels: 10 days	Spiked urine	[25]
Sandwich immunoassay with specific cAbs on COF-AuNPs-modified GCE, and dAb-decorated NiCo ₂ S ₄ @CeO ₂ microspheres as nanolabels	DPV	0.010–50/0.002	cAbs/COFs/AuNPs/GCE ~ 1 h 10 min; COFs-AuNPs: ~ 1 h 15 min; Ab ₂ /NiCo ₂ S ₄ @CeO ₂ : ~ 43 h/ ~ 15 min	cAbs/COFs/AuNPs/GCE: 7 weeks; dAb-NiCo ₂ S ₄ @CeO ₂ nanolabels: not reported	Spiked plasma	[26]
Label-free immunosensor with specific cAbs on Au-Galinstan Nd-modified disposable paper electrode	EIS	100–1000000/64	cAbs/Au-Galinstan Nds/paper electrode > 1 h; Au-Galinstan Nds: ~ 72 h/not reported	cAbs/Au-Galinstan Nds/paper electrode: 20 days	Spiked serum	[27]
Sandwich immunoassay with cAb-MBs and dAbs labeled with Strep-HRP and detection at SPCEs	Amperometry	87–7500/26	cAb-MBs: 2.2 h/45 min	cAb-MBs: 22 days	Cultured cancer cell extracts	[20]
Sandwich immunoassay based on the use of btn-cAb/Neu/p-ABA/SPCE and dAb/HOOC-CeO ₂ NPs as nanolabels	Amperometry	33–600/9.9	Assay preparation time: casein/ btn-cAb/Neu/p-ABA/SPCEs: 2 h; dAb/HOOC-CeO ₂ NPs: 3.5 days (HOOC-CeO ₂ NPs: 56 h and 27 h for dAb attachment)/test time: 60 min	btn-cAb/Neu/p-ABA/SPCE: 30 days; dAb/HOOC-CeO ₂ NPs nanolabels: 7 days	Plasma from CRC patients	This work

ABEL, aminobutylethyl isoluminol; *Ab*₂, secondary antibody; *Au-Galinstan* Nds, gold-Galinstan nanodroplets; *AuNPs*, gold nanoparticles; *BSA*, bovine serum albumin; *cAb*, capture antibody; *COFs*, covalent organic frameworks; *CRC*, colorectal cancer; *NWs*, nanowires; *dAb*, detection antibody; *DPV*, differential pulse voltammetry; *ECL*, electrochemiluminescence; *EIS*, electrochemical impedance spectroscopy; *GCE*, glassy carbon electrode; *Strep-HRP*, streptavidin horseradish peroxidase; *MBs*, magnetic beads; *MNPs*, magnetic nanoparticles; *MXene*, two-dimensional transition metal carbides/nitrides; *PdPiBP*, palladium-platinum bipyrimids; *PEI*, polyethyleneimine; *PFO*, perfluorooctanoate; *PtNPs*, platinum nanoparticles; *RGOs*, reduced graphene oxides; *SPCEs*, screen-printed carbon electrodes

The reproducibility in the preparation of the multifunctional nanolabel was checked by determining its pseudocatalytic activity using three different batches. An average value of 156 U mg^{-1} with a relative standard deviation (RSD) of 3.1% was obtained. In addition, the reproducibility of the amperometric measurements made with the developed immunoplateform was evaluated by comparing the amperometric responses for 200 pg mL^{-1} TIM-1 obtained with different immunoplateforms prepared in the same manner using the same or different nanolabel batches. The RSD values obtained, 2.9% ($n=8$) and 3.6% ($n=7$), respectively, confirmed the reproducibility of the protocols for the preparation of the nanolabel and the immunoplateform as well as for the amperometric transduction, ensuring the robust performance of the immunoplateform even when different batches of dAb/HOOC-CeO₂NPs were used.

The storage stability of the immunoplateforms and the dAb/HOOC-CeO₂NPs nanolabels once prepared was evaluated separately. This evaluation was carried out by comparing the amperometric responses obtained over time in the presence and absence of the TIM-1 standard (500 pg mL^{-1}) (Fig. S6, Supporting Information). As it can be seen, the prepared immunoplateforms provided stable responses for at least 30 days while the stability of the bionanoconjugates was 7 days.

The selectivity of the developed immunoplateform was evaluated by testing various non-target interfering proteins that might be present in biological samples. The tested interfering compounds included circulating proteins (hIgG, Hb, and HSA) as well as circulating and tumor tissue biomarkers (TNF, POSTN, and IL-13R α 2) [36–41]. These potential interferents were checked at the concentrations commonly found in biological samples. The cross-reactivity of each non-target protein was examined both in the presence (500 pg mL^{-1}) and in the absence of TIM-1. The results shown in Fig. S7 (Supporting Information) indicated that there were no significant differences in the T/B ratios in the presence of any of the tested proteins.

Analysis of plasma samples from CRC patients

The developed immunoplateform was used to analyze 16 plasma samples from healthy (H) individuals ($n=8$) and CRC patients ($n=8$), diagnosed at various stages of the disease (I–IV). Three replicates were performed for each sample, and the mean and associated error were calculated.

To evaluate the potential presence of a matrix effect, the slope value of the calibration curve constructed with TIM-1 standards ($(2.38 \pm 0.06) \text{ nA mL pg}^{-1}$) was statistically compared with those constructed in twofold diluted plasma samples of a healthy individual ($(2.4 \pm 0.1) \text{ nA mL pg}^{-1}$). Clearly, no significant differences between the slope

values, with a t_{exp} value of 1.24, which is below the critical t -value of 2.776 ($n=3$, $\alpha=0.05$), were found.

Therefore, as no apparent matrix effect was observed under these conditions, the TIM-1 concentration in the samples was determined by simple interpolation of the amperometric signals obtained for the 2-times diluted plasma samples into the calibration plot constructed with TIM-1 standards. The results, shown in Fig. 6, compare the concentrations found in H individuals with those obtained for CRC patients at different disease stages.

The results obtained for the determination of TIM-1 in this kind of sample suggested a significantly higher plasma concentration in CRC patients compared to H individuals, with an increase of TIM-1 levels in parallel to CRC progression. In this sense, CRC patients at more aggressive and metastatic stages (stages III and IV) presented higher TIM-1 plasma levels than CRC patients at non-metastatic stages of the disease (stages I and II). To evaluate the diagnostic potential of the developed bioanalytical platforms in CRC patients, and to determine the optimal cut-off values for reliable discrimination among different stages of the disease, ROC curve analysis was performed (Fig. S7, Supporting Information). These curves revealed that the cut-off value for discriminating CRC patients and H individuals was $176.45 \text{ pg mL}^{-1}$ with 100% specificity and 100% sensitivity. This cut-off value is the first one reported for TIM-1 in CRC and is similar to that claimed for clear-cell carcinoma (142 pg mL^{-1}), an uncommon salivary gland carcinoma [35].

Furthermore, the ROC curve analysis suggested the usefulness of the plasma TIM-1 contents determined with the developed immunoplateform not only for diagnosis but also for staging of CRC, through the cut-off values shown

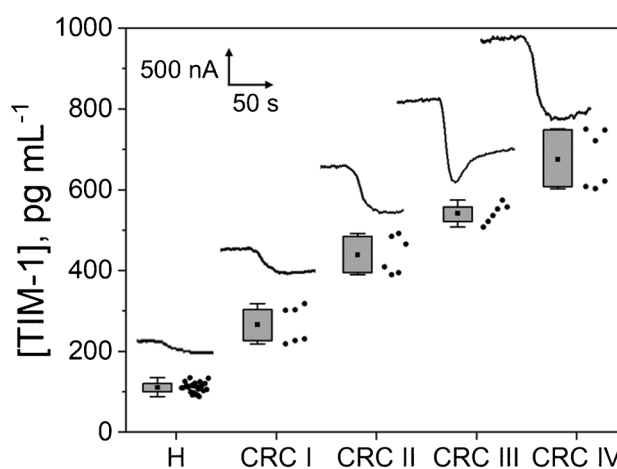


Fig. 6 TIM-1 concentrations determined with the developed immunoplateform in plasma samples grouped into pools of H individuals and CRC patients according to their stage. A representative amperometric curve for each group is also shown

in Fig. S8. These results are fully consistent with those obtained from solid through pathological anatomy and agree with CRC classification guidelines-TNM classification criteria from the American Joint Committee on Cancer. Therefore, the quantitative plasma contents provided by the developed simple and minimally invasive method in just 60 min are of great interest to add precision to the conventional diagnoses and prognoses, thus contributing to the personalized and more efficient management of patients with CRC.

Additionally, the accuracy of the results obtained with the developed immunoplatfrom for plasma analysis was evaluated through recovery studies. A representative sample from each group was spiked with 150 pg mL^{-1} of TIM-1 standard. The results obtained using the same protocol as for non-spiked samples and after subtracting the endogenous content from the total concentration measured post-spiking, yielded recovery values between 98 and 104% across the five types of analyzed samples (Table S3, Supporting Information), thus confirming the excellent accuracy of the methodology.

Conclusions

In this work, the opportunities offered by HOOC-CeO₂NPs as multifunctional nanolabels in electrochemical immunosensing have been explored. Such NPs function both as nanozymes with robust pseudo-peroxidase activity and stable nanocarriers of detector antibodies. Their use to construct an electrochemical immunosensor involving electrochemical grafting, a sandwich format, and amperometry transduction onto SPCEs, has allowed the sensitive and selective determination of TIM-1, a biomarker of emerging relevance in cancer angiogenesis. The experimental results proved the superior stability of these nanozymes (HOOC-CeO₂NPs) compared to the natural enzyme (HRP) in the presence of high concentrations of H₂O₂, the simplification in the nanolabel preparation (which, unlike previous studies, do not require their combination with other materials or their decoration with HRP molecules), and their ability to improve moderately the sensitivity in electrochemical immunosensing compared to the more conventional enzymatic labeling strategy used in sandwich immunoassays and immunosensors involving the dAb and a HRP-secondary antibody.

The developed dAb/HOOC-CeO₂NPs-assisted immunoplatfrom has provided detectability (LOD of 9.9 pg mL^{-1}) and a linear concentration range ($33\text{--}600 \text{ pg mL}^{-1}$) compatible with clinical applications and has been successfully applied to the analysis of plasma samples from cancer patients after a simple sample dilution. Interestingly, the results obtained in CRC scenarios demonstrated the potential of determining TIM-1 plasma levels both to diagnose and

to stratify this neoplasia, providing the first cut-off values described for this purpose. It is also important to highlight that, according to the state of the art, this was the first electrochemical biotool for TIM-1 that demonstrated applicability in real-world scenarios. The developed immunoplatfrom was also advantageous over conventional ELISA methodology in terms of applicability in decentralized and resource-limited settings. In addition, the opportunities offered by these multifunctional CeO₂-based nanomaterials would be transferable and exploitable in other types of biosensing.

Therefore, this work provides both novel and relevant results that highlight the value of this innovative and multifunctional label in immunosensing constituting a good starting point to continue exploiting the potential of this new hybrid nanomaterial with great versatility of modification in affinity biosensing (not only electrochemical). We envision, for example, the development of immunoplatfroms involving other formats (for instance, direct competitive configuration by immobilizing the target antigen instead of the detection antibody on CeO₂NPs) or with in other affinity systems besides immunosensors, such as nucleic acid, peptide or aptameric bioplatfroms.

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Data availability The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Declarations

Ethical approval The plasma samples from healthy and CRC patients handled and analyzed were provided by biobank of Hospital Clínico San Carlos after approval of the corresponding Ethical Committee (CEI PI 13_2020-v2).

Competing interests The authors declare no competing interests.

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References

- Campuzano S, Yáñez-Sedeño P, Pingarrón JM (2020) Electrochemical affinity biosensors based on selected nanostructures for food and environmental monitoring. *Sensors* 20:5125. <https://doi.org/10.3390/s20185125>
- Kurup CP, Ahmed MU (2023) Nanozymes towards personalized diagnostics: a recent progress in biosensing. *Biosensors* 13:461. <https://doi.org/10.3390/BIOS13040461>
- Wang Q, Liu J, He L, Liu S, Yang P (2023) Nanozyme: a rising star for cancer therapy. *Nanoscale* 15(30):12455–12463. <https://doi.org/10.1039/D3NR01976D>
- Diao Q, Chen X, Tang Z, Li S, Tian Q, Bu Z, Liu H, Liu J, Niu X (2024) Nanozymes: powerful catalytic materials for environmental pollutant detection and degradation. *Environ Sci: Nano* 11:766–796. <https://doi.org/10.1039/D3EN00844D>
- Bilal M, Khaliq N, Ashraf M, Hussain N, Baqar Z, Zdzarta J, Jesionowski T, Iqbal HMN (2023) Enzyme mimic nanomaterials as nanozymes with catalytic attributes. *Colloids Surf B* 221:112950. <https://doi.org/10.1016/j.colsurfb.2022.112950>
- Hui W, Erkang W (2013) Nanomaterials with enzyme-like characteristics (nanozymes): next-generation artificial enzymes. *Chem Soc Rev* 42:6060–6093. <https://doi.org/10.1039/C3CS35486E>
- Das B, Franco JL, Logan N, Balasubramanian P, Kim MI, Cao C (2021) Nanozymes in point-of-care diagnosis: an emerging futuristic approach for biosensing. *Nano-Micro Lett* 13:193. <https://doi.org/10.1007/s40820-021-00717-0>
- Anboo S, Yon Lau S, Kansedo J, Yap P-S, Hadibarata T, Jeevanandam J, Kamaruddin AH (2022) Recent advancements in enzyme-incorporated nanomaterials: synthesis, mechanistic formation, and applications. *Biotechnol Bioeng* 119:2609–2638. <https://doi.org/10.1002/bit.28185>
- Li F, Zou L, He J, Wu Y, Yang L, Liu Q, Wu Q, Yang X (2021) On the correlation between structure and catalytic activity of mesoporous ceria nanoparticles. *J Catal* 402:300–309. <https://doi.org/10.1016/j.jcat.2021.08.047>
- Varvoutis G, Lykaki M, Marnellos GE, Konsolakis MC (2023) Recent advances on fine-tuning engineering strategies of CeO₂-based nanostructured catalysts exemplified by CO₂ hydrogenation processes. *Catalysts* 13:275. <https://doi.org/10.3390/catal13020275>
- Centane S, Nyokong T (2023) Co phthalocyanine mediated electrochemical detection of the HER2 in the presence of Au and CeO₂ nanoparticles and graphene quantum dots. *Bioelectrochemistry* 149:108301. <https://doi.org/10.1016/j.bioelechem.2022.108301>
- Cheng W, Lin Z, Zhao L, Fan N, Bai H, Cheng W, Zhao M, Ding S (2022) CeO₂/MXene heterojunction-based ultrasensitive electrochemiluminescence biosensing for BCR-ABL fusion gene detection combined with dual-toehold strand displacement reaction for signal amplification. *Biosens Bioelectron* 210:114287. <https://doi.org/10.1016/j.bios.2022.114287>
- Centane S, Mgidlana S, Openda Y, Nyokong T (2022) Electrochemical detection of human epidermal growth factor receptor 2 using an aptamer on cobalt phthalocyanines – cerium oxide nanoparticle conjugate. *Bioelectrochemistry* 146:108146. <https://doi.org/10.1016/J.BIOELECTCHEM.2022.108146>
- Pang X, Li J, Zhao Y, Wu D, Zhang Y, Du B, Ma H, Wei Q (2015) Label-Free electrochemiluminescent immunosensor for detection of carcinoembryonic antigen based on nanocomposites of GO/MWCNTs-COOH/Au@CeO₂. *ACS Appl Mater Interfaces* 7:19260–19267. https://doi.org/10.1021/ACSAMI.5B05185/ASSET/IMAGES/LARGE/AM-2015-05185Q_0007.JPEG
- Pachauri N, Dave K, Dinda A, Solanki PR (2018) Cubic CeO₂ implanted reduced graphene oxide-based highly sensitive biosensor for non-invasive oral cancer biomarker detection. *J Mater Chem B* 6:3000–3012. <https://doi.org/10.1039/C8TB00653A>
- Wang M, Hu M, Hu B, Guo C, Song Y, Jia Q, He L, Zhang Z, Fang S (2019) Bimetallic cerium and ferric oxides nanoparticles embedded within mesoporous carbon matrix: electrochemical immunosensor for sensitive detection of carbohydrate antigen 19–9. *Biosens Bioelectron* 135:22–29. <https://doi.org/10.1016/J.BIOS.2019.04.018>
- Yu S, Zou G, Wei Q (2016) Ultrasensitive electrochemical immunosensor for quantitative detection of tumor specific growth factor by using Ag@CeO₂ nanocomposites as labels. *Sens Actuators B Chem* 222:314–320. <https://doi.org/10.1016/J.SNB.2015.08.117>
- Li Y, Zhang Y, Li F, Feng J, Li M, Chen L, Dong Y (2017) Ultrasensitive electrochemical immunosensor for quantitative detection of SCCA using Co₃O₄@CeO₂-Au@Pt nanocomposite as enzyme-mimetic labels. *Biosens Bioelectron* 92:33–39. <https://doi.org/10.1016/j.bios.2017.01.065>
- Ramos-López C, García-Rodrigo L, Sánchez-Tirado E, Agüí L, González-Cortés A, Yáñez-Sedeño P, Pingarrón JM (2024) Cerium dioxide-based nanostructures as signal nanolabels for current detection in the immunosensing determination of salivary myeloperoxidase. *Microchem J* 201:110505. <https://doi.org/10.1016/J.MICROC.2024.110505>
- Quinchia J, Blázquez-García M, Torrente-Rodríguez RM, Ruiz-Valdepeñas Montiel V, Serafín V, Rejas-González R, Montero-Calle A, Orozco J, Pingarrón JM, Barderas R, Campuzano S (2024) Disposable electrochemical immunoplatfrom to shed light on the role of the multifunctional glycoprotein TIM-1 in cancer cells invasion. *Talanta* 267:125155. <https://doi.org/10.1016/J.TALANTA.2023.125155>
- Lee SH, Lee HS, Park G, Oh SM, Oh DS (2019) Dual actions on gout flare and acute kidney injury along with enhanced renal transporter activities by Yokuininto, a Kampo Medicine. *BMC Compl Altern Med* 19:57. <https://doi.org/10.1186/s12906-019-2469-9>
- Vaidya VS, Ford GM, Waikar SS, Wang Y, Clement MB, Ramirez V, Glaab WE, Troth SP, Sistare FD, Prozialeck WC, Edwards JR, Bobadilla NA, Mefferd SC, Bonventre JV (2009) A rapid urine test for early detection of kidney injury. *Kidney Int* 76(1):108–114. <https://doi.org/10.1038/ki.2009.96>
- Chung HJ, Pellegrini KL, Chung J, Wanigasuriya K, Jayawardene I, Lee K, Lee H, Vaidya VS, Weissleder R (2015) Nanoparticle detection of urinary markers for point-of-care diagnosis of kidney injury. *PLoS One* 10(7):e0133417. <https://doi.org/10.1371/journal.pone.0133417>
- Yang H, Wang H, Xiong C, Chai Y, Yuan R (2018) Highly sensitive electrochemiluminescence immunosensor based on ABEI/H₂O₂ system with PFO dots as enhancer for detection of kidney injury molecule-1. *Biosens Bioelectron* 116:16–22. <https://doi.org/10.1016/J.BIOS.2018.05.032>
- Liu C, Yang W, Min X, Zhang D, Fu X, Ding S, Xu W (2021) An enzyme-free electrochemical immunosensor based on quaternary

- metallic/nonmetallic PdPtBP alloy mesoporous nanoparticles/MXene and conductive CuCl₂ nanowires for ultrasensitive assay of kidney injury molecule-1. *Sens Actuators B Chem* 334:129585. <https://doi.org/10.1016/J.SNB.2021.129585>
26. Boyacıoğlu H, Yola BB, Karaman C, Karaman O, Atar N, Yola ML (2022) A novel electrochemical kidney injury molecule-1 (KIM-1) immunosensor based covalent organic frameworks-gold nanoparticles composite and porous NiCo₂S₄@CeO₂ microspheres: the monitoring of acute kidney injury. *Appl Surf Sci* 578:152093. <https://doi.org/10.1016/j.apsusc.2021.152093>
 27. Das M, Chakraborty T, Lin CY, Lei KF, Kao CH (2023) Hierarchical gold-Galinstan nanodendrites modified disposable immunosensor for the label-free detection of KIM-1 by antibody immobilization on staphylococcal protein A. *Appl Surf Sci* 607:154930. <https://doi.org/10.1016/J.APSUSC.2022.154930>
 28. Chen HI, Chang HY (2005) Synthesis of nanocrystalline cerium oxide particles by the precipitation method. *Ceram Int* 31:795–802. <https://doi.org/10.1016/J.CERAMINT.2004.09.006>
 29. Such Basáñez I (2015) Inmovilización de complejos organometálicos en soportes sólidos para aplicación en catálisis. Tesis doctoral, Universidad de Alicante. <http://rua.ua.es/dspace/handle/10045/47349>. Accessed 26 Nov 2024
 30. Farahmandjou M, Zarinkamar M, Firoozabadi TP (2016) Synthesis of cerium oxide (CeO₂) nanoparticles using simple CO-precipitation method. *Rev Mex Fis* 62:496–499. http://www.scielo.org.mx/scielo.php?script=sci_arttext&pid=S0035-001X2016000500496&lng=es&nrm=iso&tlng=en. Accessed 26 Nov 2024
 31. The International Centre for Diffraction Data - ICDD. <https://www.icdd.com/>. Accessed 18 October 2024
 32. Estevez AY, Ganesana M, Trentini JF, Olson JE, Li G, Boateng YO, Lipps JM, Yablonski SER, Donnelly WT, Leiter JC, Erlichman JS (2019) Antioxidant enzyme-mimetic activity and neuroprotective effects of cerium oxide nanoparticles stabilized with various ratios of citric acid and EDTA. *Biomolecules* 9:562. <https://doi.org/10.3390/BIOM9100562>
 33. Patil S, Sandberg A, Heckert E, Self W, Seal S (2007) Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential. *Biomaterials* 28:4600–4607. <https://doi.org/10.1016/J.BIOMATERIALS.2007.07.029>
 34. Huang B, Yao C, Yang J, Dua S, Lu X (2020) A study on the electrochemical behavior of hydroquinone at a nanometer cobalt/L-glutamate modified electrode. *RSC Adv* 10:43834–43839. <https://doi.org/10.1039/D0RA07222B>
 35. Kushlinskii NE, Gershtein ES, Naberezhnov DS, Taipov MA, Bezhanova SD, Pushkar' DY, Matveev VB, Stilidi IS (2019) Kidney Injury Molecule-1 (KIM-1) in blood plasma of patients with clear-cell carcinoma. *Bull Exp Biol Med* 167:388–392. <https://doi.org/10.1007/S10517-019-04533-W>
 36. Ben QW, Zhao Z, Ge SF, Zhou J, Yuan F, Yuan YZ (2009) Circulating levels of periostin may help identify patients with more aggressive colorectal cancer. *Int J Oncol* 34:821–828. https://doi.org/10.3892/ijo_00000208
 37. Barderas R, Bartolomé RA, Fernández-Aceñero MJ, Torres S, Casal JI (2012) High expression of IL-13 receptor $\alpha 2$ in colorectal cancer is associated with invasion, liver metastasis, and poor prognosis. *Cancer Res* 72:2780–2790. <https://doi.org/10.1158/0008-5472.CAN-11-4090>
 38. Al Obeid OA, Alkhayal KA, Al Sheikh A, Zubaidi AM, Vaali-Mohammed MA, Boushey R, Mckerrow JH, Abdulla MH (2014) Increased expression of tumor necrosis factor- α is associated with advanced colorectal cancer stages. *World J Gastroenterol* 20:18390–18396. <https://doi.org/10.3748/wjg.v20.i48.18390>
 39. Li Z, Zhang X, Yang Y, Yang S, Dong Z, Du L, Wang L, Wang C (2015) Periostin expression and its prognostic value for colorectal cancer. *Int J Mol Sci* 16:12108. <https://doi.org/10.3390/IJMS160612108>
 40. Deng X, Ao S, Hou J, Li Z, Lei Y, Lyu G (2019) Prognostic significance of periostin in colorectal cancer. *Chin J Cancer Res* 31:547. <https://doi.org/10.21147/J.ISSN.1000-9604.2019.03.16>
 41. Warsinggih LF, Labeda I, Lusikooy RE, Mappincara Faruk M (2021) The relationship of tumor necrosis factor alpha levels in plasma toward the stage and differentiation degree in colorectal cancer. *Med Clín Práct* 4:100224. <https://doi.org/10.1016/J.MCPSP.2021.100224>

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