



Label-free milk biopsy using nanochannel-based biosensors for early-stage bovine mastitis screening

Deepanshu Verma^{1,2} · Hedieh Haji-Hashemi² · Beatriz Prieto-Simón^{2,3}

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Abstract

A label-free electrochemical biosensor is presented for the detection of miRNA-223, a host immune-derived biomarker upregulated in early mastitis. The biosensor consists of porous anodic alumina (pAAO) membranes featuring high-aspect-ratio porous structure, functionalized with ssDNA probes. This nanochannel-based design increases surface area for probe immobilization and enhances sensitivity by amplifying hybridization-induced changes in ionic transport.

Upon hybridization with miRNA-223 to the immobilized ssDNA probe, partial pore blockage impedes the diffusion of a redox probe added to the measuring solution. This change in diffusion is quantified via square wave voltammetry. The biosensor enables direct miRNA detection in raw milk, eliminating the need for RNA extraction or amplification.

The biosensor was systematically optimized for pore diameter, probe concentration, and blocking conditions, and demonstrated consistent signal suppression in the presence of synthetic miRNA-223 across a dynamic range from 0.1 pM to 1 nM in both phosphate buffer and 0.1% raw milk. Application to milk collected from clinically classified cows showed clear discrimination between healthy and subclinical samples, with an area under the receiver operating characteristic curve of 0.96. Statistical analysis across 30 replicate sensor readings confirmed significant group-level discrimination ($p=0.016$, Mann–Whitney U test). The biosensor's high sensitivity and specificity are attributed to its diffusion-limited pore-based architecture and stable surface functionalization, which together enable selective and reproducible hybridization responses. Its demonstrated compatibility with raw milk furthers its robustness and analytical reliability under real sample conditions. The ability to detect early-stage, immune-derived miRNAs in minimally processed milk (0.1% v/v raw milk in phosphate buffer) supports the deployment of this biosensor in on-farm, real-time surveillance systems. By eliminating reliance on pathogen detection and enabling pre-symptomatic classification, this work aims to contribute to improved livestock health management, antimicrobial stewardship, and broader one-health objectives.

Keywords Electrochemical biosensor · Square wave voltammetry · Nanochannel-based label-free sensing · Porous anodic alumina membranes · Subclinical mastitis screening · Milk biopsy · Dairy herd health monitoring

Introduction

Bovine mastitis is a major animal disease affecting dairy cattle worldwide. It causes reduced milk yield, lower product quality, increased veterinary costs, and premature culling,

contributing to both economic losses and declining animal welfare. Global losses are estimated at over US\$65 billion annually [1, 2]. In addition to farm-level burden, mastitis contributes to critical public health and environmental issues. Antibiotics are often administered to cows without microbiological confirmation of infection, especially in subclinical cases; in some settings, blanket treatments are applied at the herd level, exposing even healthy animals to antimicrobials. As a result, drug residues can persist in milk and enter the environment through animal waste, increasing the risk of antimicrobial resistance development and spread in agricultural ecosystems [3–5]. These challenges make mastitis not merely a veterinary issue, but a One Health

✉ Beatriz Prieto-Simón
bprieto@iciq.es

¹ Universitat Rovira i Virgili, Tarragona 43007, Spain

² Institute of Chemical Research of Catalonia, The Barcelona Institute of Science and Technology, Av. Països Catalans 16, Tarragona 43007, Spain

³ ICREA, Pg. Lluís Companys 23, Barcelona 08010, Spain

priority that affects animal welfare, food safety, and ecological balance.

Current diagnostic methods for mastitis, such as somatic cell count (SCC), bacterial culture, and polymerase chain reaction (PCR), have notable limitations. SCC lacks specificity, culture is time-intensive, and PCR, though being highly sensitive, is often impractical for routine use due to cost and technical requirements [6–8]. These limitations are especially problematic in detecting subclinical mastitis, which presents no visible symptoms, and thus remains underdiagnosed despite its high transmission potential [9].

To address these limitations, host response-based diagnostics have emerged as a compelling alternative to pathogen-focused assays. This approach aims to detect the physiological changes induced by infection, often earlier than pathogen detection allows. Such diagnostics primarily target host-derived biomarkers that report these perturbations. Among various of these biomarkers, microRNAs (miRNAs) are of particular interest due to their evolutionary conservation, high stability, and role in post-transcriptional gene regulation during immune responses [10]. Furthermore, their presence in multiple biofluids, including serum, saliva, and milk, makes them attractive targets for non-invasive diagnostic applications.

Specifically, miRNA-223 has been identified as a robust candidate biomarker for bovine mastitis. It plays a key role in innate immune regulation and has been shown to be upregulated during mastitis-related inflammation in both blood and milk [11, 12]. Notably, miRNA-223 is significantly overexpressed in milk during the acute phase of mastitis, supporting its diagnostic relevance in early or subclinical infections [11]. The possibility to detect miRNA-223 directly in milk offers a rapid, non-invasive “milk biopsy” approach with great potential for on-farm disease screening.

In this study, we report on the development of a label-free electrochemical biosensor for the direct detection of miRNA-223 in raw bovine milk. The platform utilizes porous anodic alumina (pAAO) membranes, a robust nanostructured material offering high surface area-to-volume ratio, versatile surface chemistry, tunable pore size, and stability during electrochemical sensing. These membranes are functionalized with amine-terminated ssDNA probes. Upon immobilized probe hybridization with the target miRNA, partial blockage of the pores occurs, restricting redox probe diffusion in the measuring solution and allowing quantification via square wave voltammetry. This diffusion-limited, pore-blocking mechanism enables sensitive, label-free detection, without the need for RNA extraction or amplification. The sensor performance was systematically optimized and validated in both buffer and 0.1% milk. When applied to raw milk samples from clinically classified cows, it successfully distinguished between healthy and subclinical cases

with high sensitivity and specificity. This approach supports the development of rapid, on-farm diagnostics that align with One Health goals by supporting improved surveillance, antimicrobial stewardship, and livestock productivity.

Materials and methods

Materials and reagents

Oxalic acid ($\geq 99\%$), phosphate-buffered saline (PBS) tablets, hydrogen peroxide (30% w/w), sodium bicarbonate (NaHCO_3), succinic acid, 3-aminopropyltriethoxysilane (APTES), ethanolamine, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dimethyl sulfoxide (DMSO), toluene, hydrochloric acid (HCl), phosphoric acid (H_3PO_4), perchloric acid (HClO_4), 2-(N-morpholino)ethanesulfonic acid (MES) buffer, copper (II) chloride dihydrate ($\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$), chromium (VI) oxide, N-hydroxysuccinimide (NHS), potassium hexacyanoferrate (III), and potassium hexacyanoferrate (II) trihydrate, were all purchased from Sigma-Aldrich (Spain) and used without further purification.

Amine-modified single-stranded DNA (ssDNA) capture probes, one complementary and another non-complementary (random sequence) to miRNA-223, used to prepare sensors and controls, respectively, along with a target miRNA-223 sequence, a synthetic ssDNA analogue of miRNA-223, two non-complementary random sequences, and a two-base mismatch sequence, were synthesized and HPLC-purified by Biosearch Technologies (UK). Full sequences and annotations are provided in Table S1 (Supplementary Information, SI).

High-purity aluminum foils (99.999%, 0.5 mm) were procured from Goodfellow Cambridge Ltd. (UK). Carbon screen-printed electrodes were purchased from DropSens (Spain). Skimmed milk powder (Millipore) was used to prepare a blocking agent solution.

Fabrication and characterization of pAAO membranes

Porous anodic alumina (pAAO) membranes were fabricated via a two-step anodization process using high-purity aluminum substrates (2 cm $\times$ 2 cm, 0.5 mm thick). The substrates were electropolished in a 1:4 (v/v) perchloric acid–ethanol solution at 20 V for 6 min with intermittent stirring.

Anodization was conducted in 0.3 M oxalic acid at 5 °C under a constant voltage of 40 V for 18 h. The resulting oxide layer was removed using a mixture of 6 wt% phosphoric acid and 1.8 wt% chromic acid at 70 °C for 1 h. A second anodization was performed under identical electrolyte and

thermal conditions in charge-controlled mode. The anodic oxide thickness was controlled by the total charge passed, using a charge density of $2.27 \text{ C}\cdot\text{cm}^{-2}$ per $\sim 1 \text{ }\mu\text{m}$ of alumina growth (i.e., $t \approx Q/(2.27)$, where t is thickness in μm and Q is the total charge density in $\text{C}\cdot\text{cm}^{-2}$) [13, 14]. Accordingly, the total charge was set to reproducibly obtain high-aspect-ratio membranes. Aluminum was etched using saturated CuCl_2 in HCl , and the barrier layer was removed using 5% phosphoric acid at $55 \text{ }^\circ\text{C}$ in a custom Teflon cell. After barrier layer removal, pAAO membranes were subjected to a pore widening process in 5% H_3PO_4 at $35 \text{ }^\circ\text{C}$ that yielded pore dissolution at $\sim 1.4 \text{ nm min}^{-1}$. Therefore, membranes were subjected to pore widening for 5 min, 15–30 min to yield pore diameters of $\sim 25 \text{ nm}$, $\sim 40 \text{ nm}$ or $\sim 60 \text{ nm}$, respectively.

The resulting pAAO membranes were used in all subsequent experiments unless otherwise specified. Membrane morphology was assessed using scanning electron microscopy (SEM). Top-view and cross-sectional images were acquired using a Scios 2 SEM (ThermoFisher Scientific) operated at 5.00 kV (100000 $\times$) and 30.00 kV (4000 $\times$), respectively.

Surface functionalization with ssDNA probes

Membranes were hydroxylated in 30% hydrogen peroxide at $99 \text{ }^\circ\text{C}$ for 30 min and subsequently dried at $60 \text{ }^\circ\text{C}$ for 2 h. Silanization was carried out using 5% (v/v) APTES in dry toluene under nitrogen atmosphere, followed by curing at $100 \text{ }^\circ\text{C}$ for 1 h. Carboxylation was performed by treating the silanized membranes with 0.2 M succinic acid (in DMSO with 0.1 M NaHCO_3 , pH 9.4) for 30 min in the dark to prevent photodegradation.

After rinsing, the carboxyl-functionalized surfaces were activated with a freshly prepared 1:1 (v/v) mixture of EDC (10 mg/mL) and NHS (15 mg/mL) in MES buffer (pH 5.4) for 30 min. The capture probes were covalently immobilized by 1 h-incubation of the activated membranes with ssDNA solutions at various concentrations (0.1 μM , 0.5 μM and 1 μM), prepared in PBS (10 mM phosphate, 137 mM NaCl , 2.7 mM KCl , pH 7.4). Unreacted active sites were quenched with 0.1 M ethanolamine in PBS for 1 h. This surface functionalization approach has been widely employed in biosensor development due to its chemical stability, effective covalent immobilization, and compatibility with porous electrochemical transducers [15, 16].

Fourier-transform infrared (FTIR) spectroscopy was employed to confirm the sequential surface functionalization steps on pAAO membranes. Spectra were acquired on a JASCO FTIR spectrometer operated in reflectance mode, with a spectral range of $4000\text{--}400 \text{ cm}^{-1}$ and a resolution of 4 cm^{-1} .

Electrochemical measurements

Electrochemical measurements were performed using an Ivium CompactStat potentiostat (Ivium Technologies, Netherlands) in a three-electrode configuration. The working electrode was a carbon screen-printed electrode with the pAAO membrane clamped over it in a custom Teflon cell. Reference and counter electrodes were inserted externally into the electroactive solution. A schematic of the setup is provided in Figure S1 (SI).

Electrochemical impedance spectroscopy (EIS) was used in characterization experiments to confirm hybridization with the target. Measurements were conducted at $+0.20 \text{ V}$ (DC bias) using a 5–10 mV AC perturbation from 100 kHz to 0.1 Hz. Nyquist plots were fitted to a Randles equivalent circuit to extract charge-transfer resistance (R_{ct}), used to assess pore blockage and surface changes post-hybridization [17, 18].

Square wave voltammetry (SWV) was used as the primary sensing technique to quantify target hybridization, applying the following parameters: -0.20 to $+0.80 \text{ V}$ potential range, 5 mV step potential, 25 mV pulse amplitude, and 5 Hz frequency.

Unless otherwise stated, both EIS and SWV were performed in 2 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ prepared in 0.01 M PBS (pH 7.4).

Biosensor response and analytical performance evaluation

Initial baseline measurements were recorded using SWV in a 2 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ solution to establish the baseline current (I_o). For target detection, 100 μL of target solution, either ssDNA analogues or miRNA-223, was incubated on the membrane surface for 40 min at room temperature under mild orbital shaking. ssDNA analogues were used during sensor development, optimization and selectivity assessment, while benchmarking/validation experiments were performed using miRNA-223 under RNase-free conditions. The choice of ssDNA during the development phase aimed to improve reproducibility during repeated incubation/handling steps, since RNA is more susceptible to degradation (e.g., driven by RNases), and thus to increasing response variability. Following incubation, the sensors were rinsed thoroughly with PBS, and SWV was performed again to obtain the post-hybridization current (I). The biosensor response was defined as normalized current change ($\Delta I/I_o$), calculated using the formula:

$$\text{Normalized current change} = \frac{I_o - I}{I_o}$$

Normalized current change was consistently used as biosensor response across optimization, calibration, selectivity, and real sample studies.

Sensing performance optimization focused on three key parameters: capture probe concentration (0.1 μM , 0.5 μM and 1 μM), hybridization time (10 to 60 min), and pore size (25 nm, 40 nm and 60 nm average diameters). Each condition was evaluated for magnitude of signal change, its consistency and reproducibility using synthetic DNA targets in PBS. Based on these optimization studies (Figures S2 and S3, and Tables S2 and S3, in SI), subsequent experiments used 40 nm pAAO's pore diameter, 0.5 μM capture probe solution to prepare sensors, and 40 min target incubation.

Calibration curves were constructed by exposing sensors to increasing concentrations of synthetic targets (0.1 pM to 1 nM) in both PBS and 0.1% milk. Each concentration was tested using three independent sensors and three control sensors prepared with a non-complementary capture probe. SWV peak currents were normalized to the baseline current and plotted as normalized current change ($\Delta I/I_0$) against the logarithm of target concentration. The theoretical limit of detection (LOD) in PBS was calculated from the calibration curve according to the IUPAC convention [19]:

$$LOD = \frac{3\sigma}{\text{slope}}$$

where σ represents the standard deviation of blank measurements. In milk, LOD was not calculated due to high background variability and nonspecific adsorption effects. This decision is supported by previous biosensing studies [20, 21], which show that the inherent heterogeneity and protein-rich composition of biological fluids such as milk, serum and saliva can introduce matrix-driven signal fluctuations. These effects make accurate and reproducible LOD estimation unreliable in the absence of extensive sample normalization or preprocessing steps, which were intentionally avoided in this study to retain assay simplicity.

The selectivity and specificity of the developed biosensor were evaluated through four experiments using different synthetic DNA targets prepared in PBS. All tests were performed at a total concentration of 1 nM unless otherwise stated. Each condition was tested in triplicates using independent sensors.

First, a fully complementary DNA sequence corresponding to miRNA-223 was used to establish the baseline hybridization response. Second, two non-complementary DNA sequences were tested individually to assess the sensor's selectivity against unrelated targets. Third, a two-base mismatch (2 bpmm) DNA strand was used to examine the biosensor's ability to discriminate closely related, non-identical sequences. Fourth, a cocktail mixture containing the

complementary and two non-complementary sequences in equal molar ratios (~ 0.33 nM each) was used to simulate a mixed environment with competing sequence interactions.

For all tests, biosensor response was calculated as normalized current change ($\Delta I/I_0$). Results were compared across groups to assess the sensor's discrimination performance. Multi-group comparisons were analyzed by one-way ANOVA with Tukey's post-hoc test; pairwise by two-tailed Student's *t*-test.

Sensor blocking and raw milk testing

When transitioning from buffer-based assays to raw milk samples, a blocking step was introduced to minimize non-specific adsorption arising from the complex biological matrix. For this purpose, skimmed milk was selected as a low-cost, protein-rich blocking agent known to reduce matrix-induced fouling in biosensor systems [22, 23]. Following ethanolamine blocking, functionalized membranes were incubated in 1% (w/v) skimmed milk in PBS for 1 h at room temperature on an orbital shaker, followed by thorough rinsing with PBS. This blocking step was applied to all sensors used in milk-based experiments.

Real sample testing was conducted using raw milk. Milk was collected from 35 milking cows from a herd of 45 Holstein-Friesian animals. Then, samples were classified as healthy or subclinical based on SCC screening (Table S4 in SI). A focused subset of five healthy and five subclinical samples was selected from the larger on-farm screened pool including cows with naturally occurring mastitis (natural pathogen exposure), without antibiotic treatment or anti-inflammatory intervention at the time of sampling. All milk samples were diluted to 0.1% in PBS prior to use.

For detection, the blocked sensors were incubated with 100 μL of diluted milk sample for 40 min at room temperature under orbital shaking. Normalized current change ($\Delta I/I_0$) was calculated based on pre- and post-incubation SWV peak currents, using the formula described previously. Statistical comparison between groups was performed using the Mann–Whitney *U* test, and Receiver Operating Characteristic (ROC) analysis was used to assess classification performance.

Results and discussion

The sensing platform developed in this study was tailored for non-invasive, on-farm detection of early-stage bovine mastitis. A pAAO-based biosensor was selected due to its high internal surface area, tunable pore dimensions, and compatibility with diffusion-limited electrochemical sensing. Detection is achieved through a label-free hybridization

mechanism in which ssDNA-functionalized pores undergo partial blockage upon binding to target miRNA-223, a host immune-derived biomarker known to be upregulated in mastitis-affected milk [11]. The sensing response arises from a combination of steric hindrance and electrostatic repulsion between the negatively charged DNA–RNA (or DNA–DNA) duplexes and the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ redox probe added to the measuring solution, which restricts ion transport through the pores. This partial blockage allows hybridization events to be transduced as measurable changes in peak current via SWV, even in raw milk samples without the need of RNA extraction or amplification. Sensor performance was subsequently evaluated through structural characterization, analytical optimization, calibration studies, selectivity testing, and analysis of raw milk.

Morphological, chemical and electrochemical characterization of pAAO sensor

First, the surface morphology of the fabricated pAAO membranes was assessed by SEM. Top-view SEM imaging (Fig. 1a) revealed highly ordered pores with an average diameter of 44.4 ± 2.7 nm and an interpore distance of 100 ± 5 nm, consistent with oxalic acid anodization at 40 V. The observed quasi-hexagonal pore arrangement reflects the self-organized nature of the anodization process [24, 25].

Cross-sectional SEM imaging (Fig. 1b) confirmed the formation of vertically aligned pores with a membrane thickness of 23.5 ± 1.5 μm . These high-aspect-ratio pores offer a large internal surface area for probe immobilization and act as confined diffusion channels, where hybridization-induced obstruction significantly limits ionic access to the electrode, thus enabling sensitive diffusion-limited electrochemical detection [26].

Next, surface functionalization of the pAAO membranes was validated by FTIR. As shown in Fig. 2, peaks corresponding to Al–O–Al framework and O–H stretching after successful hydroxylation appear at ~ 600 – 900 cm^{-1} and 3000 – 3400 cm^{-1} , respectively. Silanization with APTES was confirmed by the appearance of characteristic C–H stretching bands (2855 – 2925 cm^{-1}) together with Si–O–Si/Si–O–Al signals near 1100 cm^{-1} , consistent with previous reports on aminosilane-modified surfaces [16]. Subsequent treatment with succinic acid introduced carboxyl functional groups, evidenced by a strong C = O stretch (~ 1720 cm^{-1}) and the COO[−]/COOH dimer envelope around ~ 1400 cm^{-1} . DNA immobilization was validated by the appearance of phosphate backbone vibrations (PO₂[−] asymmetric ~ 1235 cm^{-1} , symmetric ~ 1088 cm^{-1}) and broad base-related absorptions in the 1700 – 1500 cm^{-1} region [27, 28].

Beyond morphological and chemical validation of the functionalized membranes, the sensing mechanism of the sensor built integrating these membranes was evaluated electrochemically. EIS was employed to probe hybridization-induced changes in charge-transfer resistance (*R*_{ct}), thereby confirming the functional role of the immobilized probes in diffusion-limited detection.

As shown in Fig. 3, the Nyquist plot recorded after target hybridization exhibited a measurable increase in mean *R*_{ct}, from 1.48 ± 0.39 k Ω to 1.98 ± 0.58 k Ω ($n=3$), corresponding to a $\sim 30.7\%$ rise. This increase reflects a hybridization-induced pore-blocking effect, where the duplex formation (DNA–DNA or DNA–RNA) leads to enhanced steric hindrance and electrostatic repulsion, impeding the access of negatively charged redox species ($[\text{Fe}(\text{CN})_6]^{3-/4-}$) to the electrode surface, shown as a decrease of the electron transfer between them. The system was modeled using a modified Randles equivalent circuit (inset in Fig. 3) comprising a solution resistance (*R*_Ω), charge-transfer resistance (*R*_{ct}),

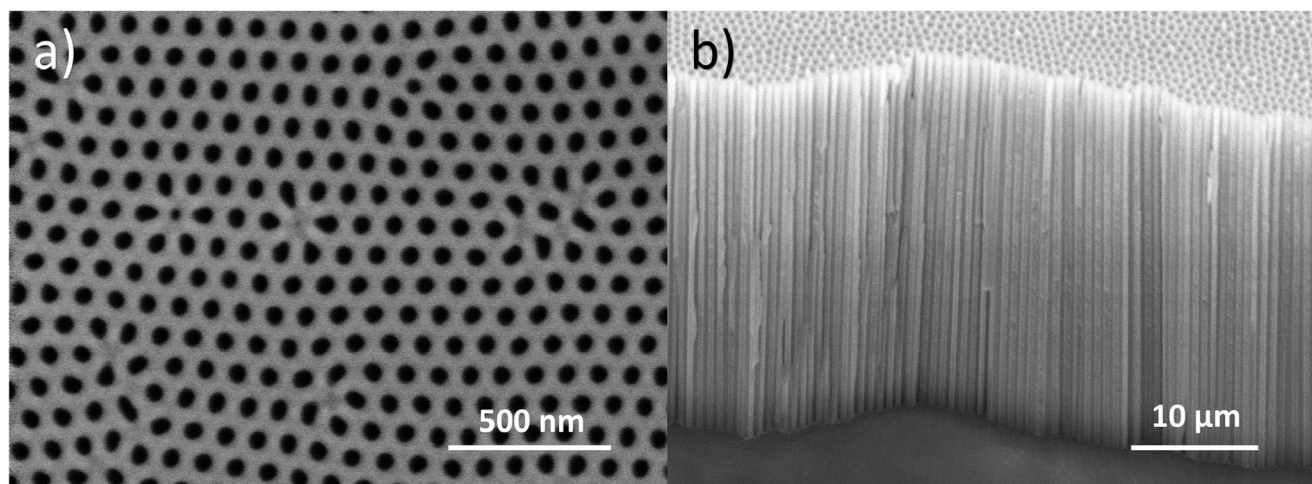


Fig. 1 Morphological characterization of pAAO membranes fabricated in 0.3 M oxalic acid at 40 V via (a) top-view SEM imaging, showing ordered pores, and (b) cross-sectional SEM imaging, highlighting vertically aligned pores

Fig. 2 Chemical characterization of pAAO membranes at different functionalization stages via FTIR, including spectra of bare (black, bottom), hydroxylated (red), silanized (green), carboxylated (blue), and DNA-conjugated (black, top) membranes

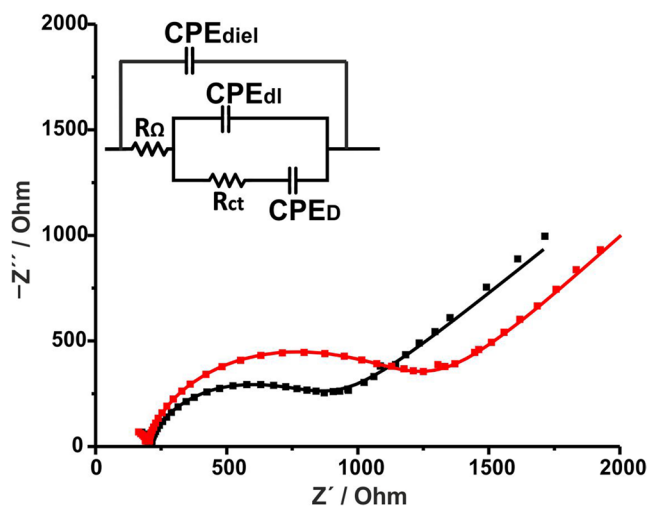
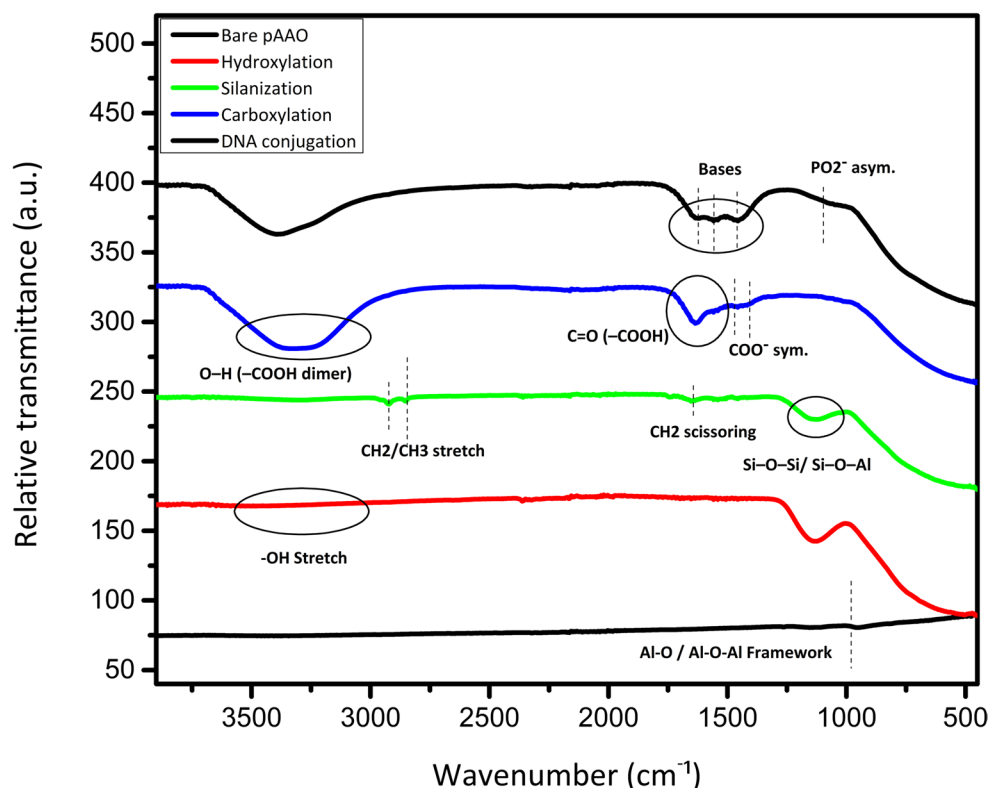


Fig. 3 Electrochemical characterization of pAAO biosensor illustrating Nyquist plots obtained from EIS measurements before (black) and after (red) target hybridization, with equivalent circuit fitting. Inset: Equivalent circuit used for EIS data fitting

and three constant phase elements (CPE). CPE_{diel} accounts for the high-frequency dielectric capacitance of the platform, CPE_{dl} for the double-layer capacitance, and CPE_D for the non-linear mass-transfer at low frequencies. CPE elements were chosen as being more representative to the frequency dispersion of the platform (non-ideal capacitive and mass-transfer behavior) typical of porous and rough surfaces.

The observed impedance behavior confirms a diffusion-limited sensing mechanism based on steric and electrostatic modulation of ionic transport, aligning with earlier findings using pAAO membranes for label-free biosensing applications [29, 30].

Optimization of sensor parameters

To ensure robust and reproducible performance, key experimental variables were systematically optimized using SWV as the primary readout method. These included pAAO pore diameter, capture probe concentration, hybridization time, and surface blocking strategy. Initial tests were conducted using synthetic ssDNA analogues due to their enhanced stability and handling consistency relative to miRNA targets [31]. A wide concentration range (0.1 pM to 1 μ M) was initially explored to evaluate sensor behavior across low and high target abundance. For these experiments, the response slope was calculated over two distinct sub-ranges: 0.1 pM–1 nM (low concentration range relevant for early-stage mastitis detection) and 1 nM–1 μ M (higher concentrations with potential signal saturation).

Among the tested pore diameters (~ 25, ~ 40 and ~ 60 nm), the sensors prepared with ~ 40 nm-pore pAAO membranes showed the most favorable performance in the 0.1 pM–1 nM range, with the steepest calibration slope (0.019) and high linearity ($R^2 = 0.97$), indicating superior sensitivity (Figure S2 and Table S2 in SI). The sensors prepared with

~ 25 nm-pore membranes, while yielding a slightly steeper slope (0.025), produced elevated background signals in control sensors, likely due to increased non-specific adsorption in smaller pores which compromises selectivity. In contrast, the sensors prepared with ~ 60 nm-pore membranes exhibited reduced slope and linearity, possibly due to insufficient confinement, leading to reduced signal modulation of diffusion-limited transport [32].

Next, three capture probe concentrations (0.1 μM , 0.5 μM and 1 μM) to prepare the sensors were evaluated. A 0.5 μM capture probe concentration was selected as the optimum, providing sensors with maximized response, without oversaturating the surface (see Table S3 in SI). Finally, target incubation time was assessed over a 1-hour period. A 40-min incubation time was found to be sufficient for signal plateauing (see Figure S3 in SI). Optimized conditions were subsequently validated using miRNA to ensure biological relevance.

Specificity, selectivity and interference analysis

To rigorously evaluate biosensor performance under realistic analytical conditions, we assessed the specificity, selectivity, and interference resistance of the biosensor comparing its response towards a DNA analogue corresponding to miRNA-223 to that obtained in the presence of various DNA interfering sequences. Selectivity was assessed by comparing the response of sensors exposed to the fully complementary DNA target (1 nM) against that obtained when incubating two non-complementary DNA sequences (RS1 and RS2). As shown in Fig. 4a, the specific DNA target produced a strong biosensor response ($\Delta I/I_0 = 0.44 \pm 0.04$), while non-complementary DNA sequences produced low but measurable background responses ($\Delta I/I_0 = 0.065 \pm 0.02$ and 0.09 ± 0.008 , for RS1 and RS2, respectively; n

= 3). Nonetheless, the signal measured upon RS1 and RS2 incubations remained substantially below the biosensor response to the complementary DNA target under the tested conditions ($p < 0.001$, one-way ANOVA with Tukey's post-hoc test), and in line with the background signal of controls mainly attributed to nonspecific adsorption of the target (confirmed by the concentration-dependent trend of control sensors, see Figure S4 in SI) [33]. Next, specificity was examined using a two-base mismatch variant of the DNA analogue of miRNA-223 at 1 nM [34, 35], which produced a partial response ($\Delta I/I_0 = 0.16 \pm 0.02$), significantly lower than the fully matched target (Fig. 4b), demonstrating the sensor's ability to discriminate closely related sequences based on mismatch sensitivity. Finally, the interfering effect caused by various mixed DNA sequences was assessed by comparing the biosensor response obtained from a 0.33 nM solution of the fully complementary target alone and that from a cocktail solution containing an equimolar mixture of the target and two non-complementary sequences (RS1 and RS2), each at ~ 0.33 nM (total concentration: 1 nM). RS1 and RS2 correspond to two non-target bovine miRNA sequences previously reported as mastitis biomarkers (see Table S1), used here as biologically relevant off-target controls [10, 36]. Both conditions yielded comparable biosensor response ($\Delta I/I_0 \sim 0.14$), indicating that the sensor selectively hybridizes with the complementary strand even in competitive environments (Fig. 4c). All group comparisons were statistically significant ($p < 0.001$, one-way ANOVA followed by Tukey's post-hoc test), validating the biosensor's ability to differentiate the response from unrelated targets, point mutations and complex sample compositions.

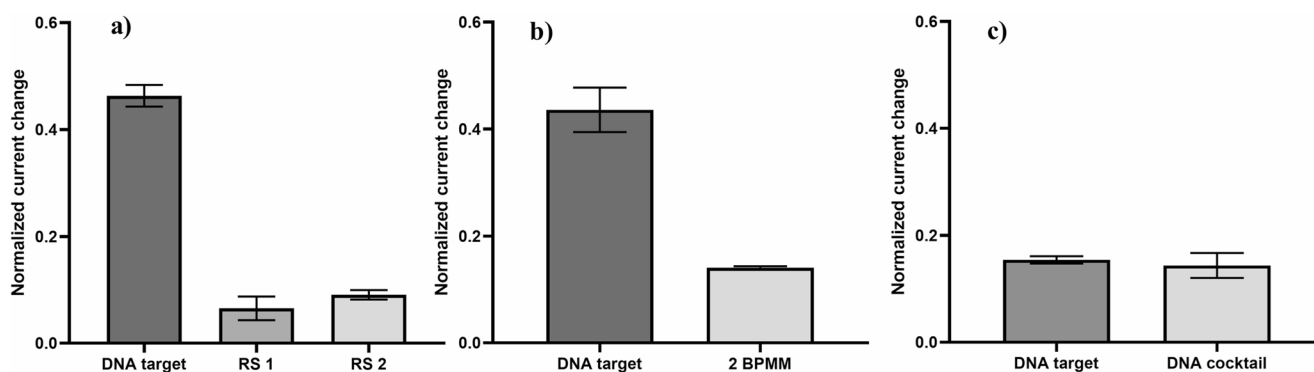


Fig. 4 (a) Selectivity test comparing the response of sensors exposed to fully complementary DNA target (1 nM) and two non-complementary DNA sequences (RS1 and RS2) (1 nM each). (b) Specificity test using a two-base mismatched (2 bpmm) DNA sequence at 1 nM, compared to the fully complementary DNA target. (c) Interference test comparing the sensor response to 0.33 nM complementary DNA target alone

and to an equimolar mixture including the target and two non-complementary DNA sequences (total 1 nM). Electrochemical measurements were performed via SWV in 2 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ prepared in 0.01 M PBS (pH 7.4). All bars represent mean $\pm$ SD ($n=3$). *** $p < 0.001$ by one-way ANOVA with Tukey's post-hoc test

Comparison of ssDNA and miRNA target sensing

Following optimization of pore diameter, probe concentration, and incubation time, the biosensor's performance was evaluated using both ssDNA analogue and RNA target, both with sequences equivalent to that of miRNA-223. Two independent sets of sensors were prepared: one tested with ssDNA and the other with RNA, each in triplicate across serial dilutions (0.1 pM to 1 nM).

As shown in Fig. 5, both DNA and RNA targets produced clear, concentration-dependent normalized current change. The biosensor response exhibited excellent linearity with respect to $\log[\text{target}]$, with R^2 values of 0.98 for both DNA and RNA fits. The linear regression equations were: $y = 0.042x + 0.65$ and $y = 0.033x + 0.59$ for DNA and miRNA, respectively. While overall responses were highly comparable, the RNA signals were consistently $\sim 7\text{--}10\%$ lower than those of DNA across the concentration range. This modest reduction aligns with established thermodynamic trends: DNA–RNA duplexes typically exhibit lower hybridization affinity than DNA–DNA counterparts under identical conditions [37, 38]. The difference is attributed to backbone asymmetry introduced by the 2'-OH group in RNA, altered base stacking, and electrostatic differences in confined sensing environments [39]. The comparable concentration-dependent trends observed for miRNA-223 and its ssDNA analogue support the transferability of the optimized assay conditions to the RNA target within the same hybridization-driven pore-blocking mechanism, with the observed differences consistent with established thermodynamics of RNA–DNA hybrid duplexes [34, 40].

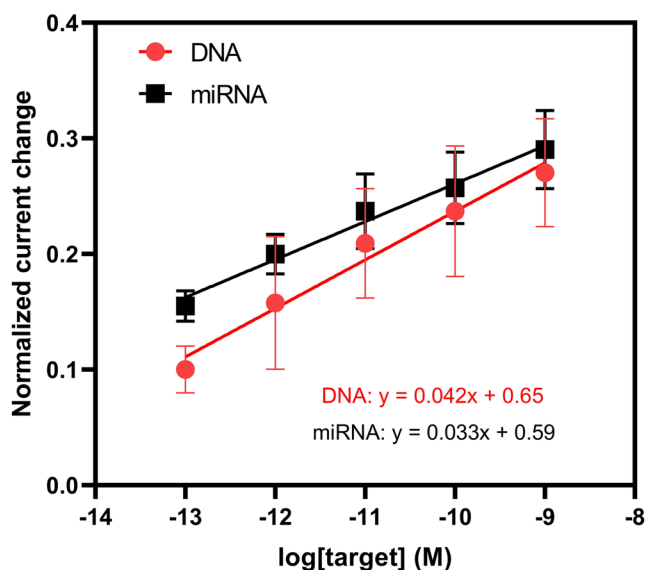


Fig. 5 Calibration plots of biosensor response to synthetic ssDNA analogues (red) and miRNA-223 RNA targets (black) over the concentration range 0.1 pM–1 nM. Data represent mean $\pm$ SD ($n=3$). Linear regression fits are shown with corresponding equations

Sensor performance in milk matrix

To evaluate sensor applicability to the analysis of complex biological matrices, sensing experiments were conducted using 0.1% (v/v) raw milk (collected from a healthy cow) diluted in PBS. The sample was spiked with synthetic miRNA-223 across a concentration range of 0.1 pM to 1 nM. Initial trials were performed without any surface blocking to assess baseline sensor behavior in milk. Under these conditions, the response of sensors and controls towards miRNA-223 spiked in diluted milk was compared. As shown in Fig. 6a, controls exhibited saturation in biosensor response after repeated miRNA-223 incubation steps, likely due to non-specific adsorption of proteins, and other macromolecules present in the milk matrix, on the sensor surface. In contrast, the response from target-specific sensors remained distinguishable across the same miRNA-223 concentration range. This fouling phenomenon is widely reported in biosensing applications involving unprocessed biological fluids, where it can significantly compromise analytical sensitivity and specificity [41, 42]. To mitigate these effects, the surface of both sensors and controls was blocked prior sensing through a 1-hour incubation of a 1% (w/v) skimmed milk solution prepared in PBS. Post-blocking, both sensors and controls were evaluated in parallel under identical conditions. As shown in Fig. 6b, the response of blocked sensors remained clearly concentration-dependent, while blocked controls showed significantly reduced response, confirming successful reduction of nonspecific adsorption [42, 43]. The reduction of control responses after blocking indicates that matrix-driven nonspecific adsorption in milk can be mitigated while maintaining target-specific signal modulation.

The calibration curve of normalized current change versus $\log[\text{miRNA-223}]$ plotted for the blocked sensor (Fig. 6c) demonstrated linear response across the full miRNA-223 concentration range (0.1 pM to 1 nM), with a slope of 0.067 and $R^2 = 0.99$. In contrast, controls exhibited lower and less consistent responses without a clear linear trend. These findings confirm that skimmed milk blocking, combined with the nanoporous architecture and the diffusion-limited sensing mechanism enabled by pAAO, supports effective biosensing performance even in raw milk matrices. The use of milk, and that of similar protein-based blocking agents (e.g., BSA or casein) has been shown to reduce non-specific adsorption and improve signal reproducibility in biosensing studies involving serum, saliva and cell lysates [43]. While the endogenous concentration of miRNA-223 was not determined in this study, the biosensor's ability to detect sub-nanomolar levels of miRNA-223 spiked in diluted raw milk highlights its potential for non-invasive mastitis diagnostics.

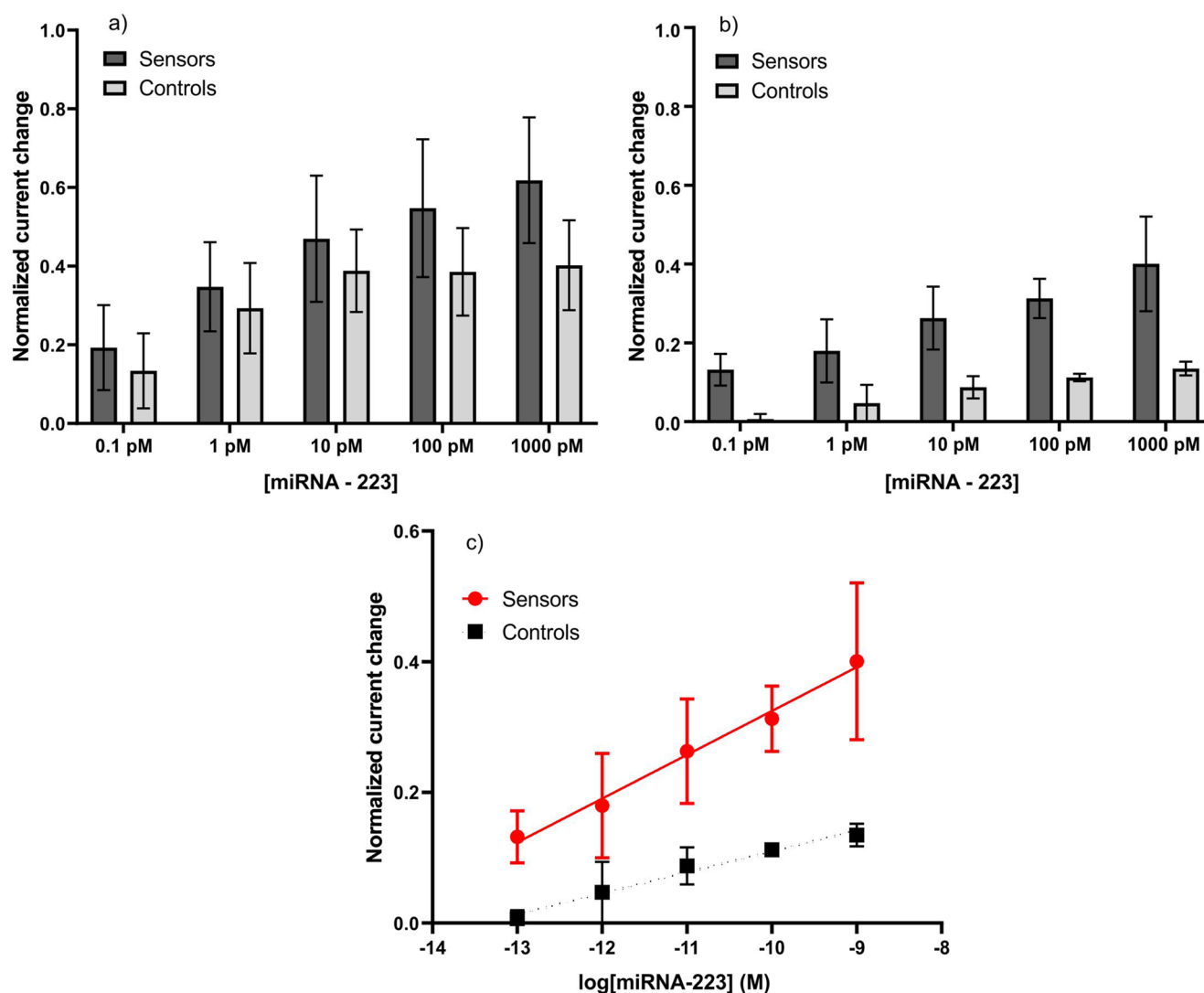


Fig. 6 Biosensor response to increasing concentrations of miRNA-223 (0.1 pM–1 nM) spiked in 0.1% raw milk diluted in PBS. (a) Unblocked sensors functionalized with complementary capture probes and controls with non-complementary probes. (b) Sensors and con-

trols blocked by 1-hour incubation in 1% skimmed milk prior to testing. (c) Calibration curves of blocked sensors and controls plotted as normalized current change versus log[miRNA-223]. Data represent mean $\pm$ SD ($n=3$)

Analysis of raw milk for the prompt identification of subclinical mastitis

miRNA-223 has been confirmed as a reliable biomarker for bovine mastitis, with multiple transcriptomic and small RNA profiling studies reporting its consistent upregulation in milk during subclinical and clinical infection states. Its stable presence in milk enables a non-invasive “milk biopsy” approach for early diagnosis [36].

To evaluate the biosensor’s diagnostic potential, raw milk samples from healthy cows and cows suffering subclinical mastitis ($n=5$ per group) were tested under optimized conditions, using three independently prepared sensors per sample. Samples were initially classified according to SCC thresholds, with values exceeding 200000 cells/

mL categorized as subclinical in line with epidemiological thresholds used in dairy herd screening (see Table S4 in SI) [44]. Importantly, these samples were collected directly from a dairy herd, reflecting naturally occurring mastitis arising from natural pathogen exposure (i.e., non-induced infection), which provides a clinically realistic test matrix for early screening. Within the screened herd (45 Holstein–Friesian animals; 35 milking cows sampled), only a limited number of subclinical cases meeting the SCC criteria were identified along the sampling window, and therefore a focused set of five subclinical and five healthy samples was selected for this assessment.

As shown in Fig. 7a, subclinical samples consistently exhibited greater normalized current change than healthy controls, with minimal overlap between groups. Only one

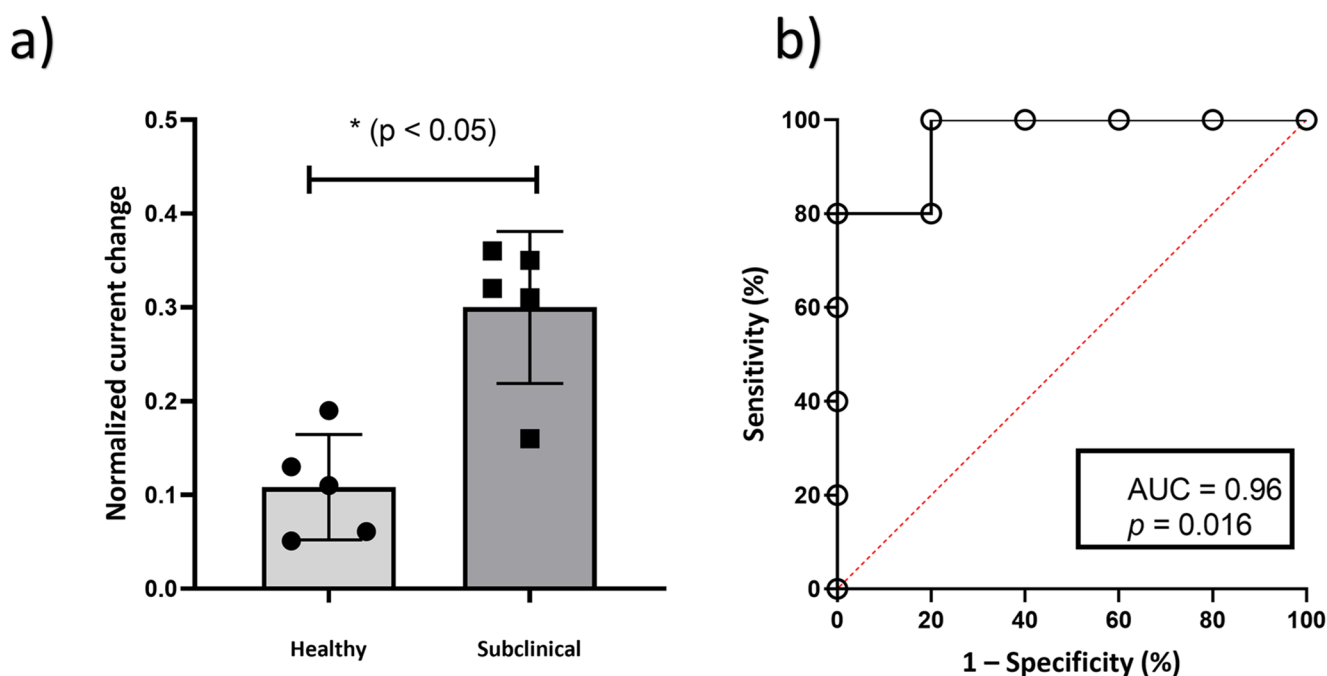


Fig. 7 Performance of the pAAO biosensor in raw milk. **(a)** Per-sample mean normalized SWV responses for milk collected from healthy cows ($n=5$) and cows identified with subclinical mastitis ($n=5$), each

sample measured with three independently fabricated sensors. **(b)** ROC curve computed from the per-sample means

subclinical sample, which had the lowest SCC in its group (258000 cells/mL, just above the 200000 cells/mL diagnostic threshold) yielded borderline values within the upper range of the healthy group. This outcome likely reflects biological variability in early-stage or borderline subclinical cases and highlights the sensor's sensitivity in detecting subtle molecular differences. A group-level statistical analysis using the Mann–Whitney U test on per-cow average responses ($n=5$ per group) confirmed a statistically significant difference ($p=0.016$, $U=1$), supporting the sensor's discriminatory capability.

To quantify classification performance, a Receiver Operating Characteristic (ROC) curve was constructed (Fig. 7b), yielding an area under the curve (AUC) of 0.96 supporting the discrimination between subclinical and healthy milk in this cohort. Using a decision threshold that maximized the separation between groups (Youden's J), the confusion matrix delivered: true positives – 4/5; false negatives – 1/5; true negatives – 5/5; false positives – 0/5. At an optimal threshold of -0.30, the biosensor demonstrated 80% sensitivity and 100% specificity to differentiate the milk from subclinical cases and healthy controls. In comparison, an SCC threshold of 200000 cells/mL shows modest mastitis diagnostic accuracy of about 44% sensitivity and 87% specificity for “any pathogen,” and 65% to 73% for major pathogens in cow-level analyses [44, 45]. Conventional bacteriological culture varies widely by pathogen and sampling scheme, with sensitivities ~ 59–98% and specificities

~ 48–97%. Moreover, time-to-result is typically 24–48 h [44, 46, 47]. PCR-based assays detect additional cases that are culture-negative, with reports indicating ~ 43–47% more positives than culture alone and, in some implementations, report very high sensitivity/specificity but require centralized labs and careful handling due to inhibitors and the risk of detecting non-viable DNA [44]. Within this context, the developed label-free miRNA-223 biosensor achieved 80% sensitivity and 100% specificity in a small cohort ($n=10$), with an AUC = 0.96, indicating competitive discrimination relative to SCC- and culture-based diagnostic methods, and approaching the classification performance of PCR, while operating as a rapid, extraction-free test.

These findings are consistent with transcriptomic studies reporting elevated levels of miRNA-223 in bovine milk during the acute phase of mastitis [11]. miRNA-223 has also been widely linked to immune activation and inflammatory processes in the mammary gland, reinforcing its relevance as a biomarker for infection-related changes [48]. Importantly, this analysis was performed using minimally processed raw milk diluted to 0.1%, without RNA extraction, purification or amplification. Hence, results underpin the sensor's robustness under near-point-of-care conditions. The ability to classify subclinical and healthy states based on direct detection of circulating miRNAs in milk highlights the potential of this platform for rapid, low-cost screening of subclinical mastitis in dairy herds. Nonetheless, the study is limited by cohort size ($n=10$), with discrimination

demonstrated for SCC-defined groups; larger validation cohorts and pathogen-resolved stratification will be required for definitive clinical performance estimates.

Conclusion

This work presents a label-free nanochannel-based electrochemical biosensor engineered for the direct detection of a host immune-derived biomarker, miRNA-223, in raw bovine milk. Leveraging pAAO membranes with covalently bound ssDNA probes complementary to miRNA-223, placed on a carbon-based transducer, the biosensor enables sensitive detection based on hybridization-induced pore blocking, without the need for signal amplification or RNA extraction. By targeting an inflammation-responsive miRNA released into milk during subclinical mastitis, the approach offers a non-invasive “milk biopsy” strategy for early disease diagnosis.

Through systematic optimization of structural and biochemical parameters, the sensor demonstrated robust concentration-dependent responses in both buffer and raw milk matrices. Indeed, by preserving its sensing performance in complex biological samples, the biosensor meets a key criterion for on-site diagnostics, supporting translational potential for early mastitis screening.

Application of the biosensor to raw milk samples from clinically classified cows showed clear discrimination between healthy and subclinical groups, with an AUC of 0.96 and statistically significant group-level differences ($p=0.016$, Mann–Whitney U test). These findings highlight the sensor’s capability to detect early-stage, host immune-derived biomarkers in untreated milk, enabling pre-symptomatic screening under minimally processed conditions.

From a broader perspective, this approach directly addresses limitations in conventional mastitis diagnostics, which are either pathogen-focused or reliant on symptomatic manifestations. By targeting host-derived biomarkers such as miRNAs in milk, which is a readily accessible, non-invasive matrix, this work aligns with One Health objectives through prompt diagnosis key for reducing antibiotic misuse, promoting sustainable disease management, and improving farm profitability [49, 50].

This proof-of-concept study establishes a diffusion-limited, label-free miRNA sensing strategy that operates in a relevant raw milk matrix and enables prompt discrimination of SCC-defined healthy versus subclinical samples collected from a herd under natural pathogen exposure. Future efforts will focus on expanding the sample set for clinical validation, assessing specificity across diverse pathogens and physiological conditions, evaluating sensor performance under on-farm operational conditions, and integrating the

sensor into portable or in-line diagnostic devices for real-time, on-farm deployment. Furthermore, multiplexing capabilities may be explored to simultaneously detect multiple biomarkers and support differential diagnosis of mastitis etiology. Together, these developments represent a step toward low-cost, accessible biosensing platforms that support proactive and precise animal health management.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Competing interests The authors declare no competing interests.

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