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# Fluorescent and electrochemical detection of nuclease activity associated with *Streptococcus pneumoniae* using specific oligonucleotide probes†

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*Streptococcus pneumoniae* (*S. pneumoniae*) represents a significant pathogenic threat, often responsible for community-acquired pneumonia with potentially life-threatening consequences if left untreated. This underscores the pressing clinical need for rapid and accurate detection of this harmful bacteria. In this study, we report the screening and discovery of a novel biomarker for *S. pneumoniae* detection. We used *S. pneumoniae* nucleases as biomarker and we have identified a specific oligonucleotide that works as substrate. This biomarker relies on a specific nuclease activity found on the bacterial membrane, forming the basis for the development of both fluorescence and electrochemical biosensors. We observed an exceptionally high sensitivity in the performance of the electrochemical biosensor, detecting as low as $10^2$ CFU mL$^{-1}$, whereas the fluorescence sensor demonstrated comparatively lower efficiency, with a detection limit of $10^6$ CFU mL$^{-1}$. Moreover, the specificity studies have demonstrated the biosensors' remarkable capacity to identify *S. pneumoniae* from other pathogenic bacteria. Significantly, both biosensors have demonstrated the ability to identify *S. pneumoniae* cultured from clinical samples, providing compelling evidence of the potential clinical utility of this innovative detection system.

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## Introduction

*Streptococcus pneumoniae* (*S. pneumoniae*) is a respiratory pathogen that disproportionately affects young children and the elderly, resulting in significant morbidity and mortality due to high rates of pneumonia,[1] bacteremia,[2] and meningitis.[3] Notably, *S. pneumoniae* is the leading cause of community-acquired pneumonia in clinical practice.[4–6] The accurate and timely identification of this pathogen is crucial for selecting appropriate clinical treatments and developing effective strategies.[7] However, the current methods employed for detecting *S. pneumoniae* rely on phenotypic characteristics, including culture-based, microscopy-based, and biochemical identification techniques.[8–11] Unfortunately, culture-based methods often entail lengthy growth and identification periods, leading to delayed positive identification and potentially unfavorable outcomes for infected individuals.[8] Consequently, there is an urgent need to develop rapid and precise approaches to identify *S. pneumoniae*.[12–14] In recent decades, several non-culture methods have emerged as alternatives for detecting *S. pneumoniae*, such as mass spectrometry,[15,16] immunoassay,[17,18] and PCR-based assays.[19,20] These methods offer significant time saving compared to traditional culture-based approaches. Nevertheless, the need of skilled technicians and sophisticated equipment may limit their availability globally. Furthermore, the search for bacterial biomarkers that allow the development of alternative detection methods continues.[21]

Herein, we report on a novel biomarker for specific identification and targeting of *S. pneumoniae* using the nuclease activity associated with these bacteria. Nuclease activity has been proposed as an attractive biomarker for bacterial

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identification,[22,23] leveraging the catalytic function of nucleases to increase the sensitivity by degrading oligonucleotide substrates. This operational concept has been effectively applied to identify bacteria, cancer cells, and various human conditions.[24–26] It is crucial to highlight that the ability to detect nucleases through oligonucleotide substrates relies on two key factors: (i) the specificity and variability of nucleases across all living organisms,[27] and (ii) the effectiveness of chemically modified nucleotides strategically positioned to customize the oligonucleotide substrate for a particular target nuclease.[28]

Previously, we have shown that nuclease activity can be used to specifically target bacteria belonging to both Gram-positive[29] and Gram-negative groups.[30] These studies used oligonucleotides that were modified at the ends with a fluorophore and quencher.[29] This fluorescent strategy allowed us to detect the degradation of the oligonucleotides, which served as a method to identify the activity of specific nucleases that can be associated with a given bacteria.[29] In this study, we have identified a specific oligonucleotide sequence that selectively recognizes the nuclease activity associated with *S. pneumoniae*. Furthermore, we have successfully developed fluorescent and electrochemical biosensors using the P3 oligonucleotide sequence as a proof-of-concept (Fig. 1). This showcases the feasibility of accurately targeting *S. pneumoniae* through its nuclease activity profile, which serves as a biomarker.

## Results and discussion

### Screening of nuclease activity associated with *S. pneumoniae*

To investigate the nuclease activity associated with *S. pneumoniae*, we began by incubating bacterial cultures with a library of oligonucleotide probes comprising DNA and RNA sequences that were modified with a fluorophore and quencher. Table S1† provides the list of oligonucleotide sequences used in this study. This fluorescent strategy offers a versatile approach that can be easily and accurately employed to measure nuclease activity. We analyzed both, the supernatants (liquid portion) of bacteria cultures and bacteria cells in suspension to differentiate between secreted and membrane-bound nucleases, respectively. In the first screening (Fig. 2a), we observed that both the supernatant and cells in suspension displayed more efficient degradation of the RNA-based probes compared to DNA probes. Notably, the probes containing RNA (mix of 4 nucleosides), RNA-PolyA, and

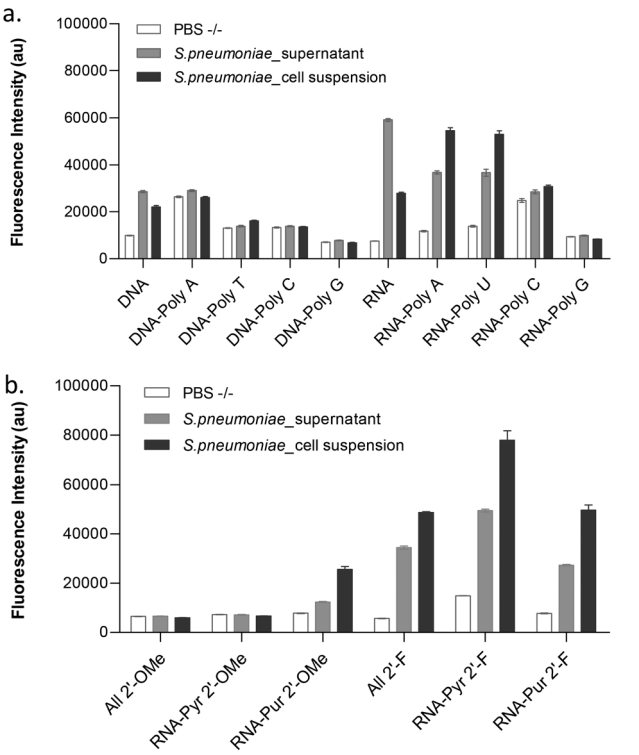


**Fig. 2** Assessment of targeted nuclease activity for *S. pneumoniae*. (a) Screening of *S. pneumoniae* bacteria cultures utilizing a library comprising DNA and RNA fluorescent probes, and (b) screening employing chimeric libraries, wherein RNA is combined with chemically modified nucleosides, specifically 2′-*O*-Methyl or 2′-Fluoro. All the experiments were carried out by triplicates (mean; bars ± SD; error bars).

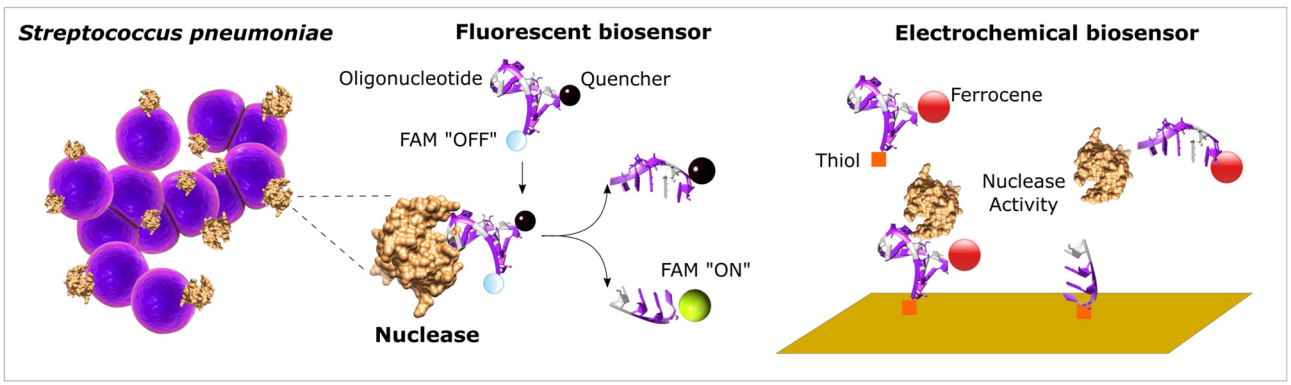


**Fig. 1** Representation of nucleases anchored to the membrane of *S. pneumoniae*, and the detection of oligonucleotide probes through fluorescence and electrochemical biosensor.


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RNA-PolyU showed the highest degradation rates. These findings indicate that the presence of nuclease activity is primarily based on RNases, indicating a potential avenue for developing tailored oligonucleotide probes for targeting *S. pneumoniae*. In the subsequent screening, we synthesized chimeric oligonucleotides by combining RNA with chemical modifications (2′-*O*-Methyl and 2′-Fluoro). The reasoning behind this approach was to bolster stability against non-specific nucleases present in other bacteria. This is essential because RNA unmodified probes are susceptible to degradation by nucleases from other bacteria, as well as endogenous mammalian nucleases.[31] Choosing an oligonucleotide sequence with high specificity for a particular nuclease demands careful consideration of both the optimal sequence and chemical nucleoside modifications. The selected sequence should be highly susceptible to degradation by the target nuclease while inducing minimal degradation by any other non-specific nucleases. This dual requirement ensures not only the effective recognition and degradation by the target nuclease but also prevents interference from non-specific nucleases, thereby enhancing the overall specificity of the oligonucleotide sequence.[32] As previously described, we conducted the second screening using chimeric oligonucleotides that combined purines of RNA with chemically modified pyrimidines (RNA-Pyr2′-OMe and RNA-Pyr2′-F), and *vice versa* (RNA-Pur2′-OMe and RNA-Pur2′-F), along with fully modified oligonucleotides (All 2′-OMe or All 2′-F).[32] Fig. 2b illustrates that the 2′-*O*-Methyl modification significantly reduced nuclease activity for All 2′-OMe and RNA-Pyr-2′-OMe probes, while the RNA-Pur-2′-OMe probe showed limited degradation, specifically by the nucleases present on the bacterial membrane. In contrast, the chimeric oligonucleotides containing 2′-Fluoro modification were well tolerated by the nuclease activity associated with *S. pneumoniae*, particularly the membrane-bound nucleases exhibited the highest degradation efficiency. These results suggest that the nuclease activity profile of *S. pneumoniae* favors RNA as the natural substrate, with limitations in degrading the 2′-*O*-Methyl modification. Interestingly, the 2′-Fluoro modification was readily degraded, presenting an opportunity for designing oligonucleotide probes to specifically target *S. pneumoniae*.

### Design of tailored oligonucleotide probes for *S. pneumoniae* and specificity studies

Based on the screening results obtained in this study, we have designed three potential oligonucleotide probes for the specific targeting of *S. pneumoniae*. The candidate oligonucleotides were designed by having the recognition moiety in the center with a mixture of 2′-Fluoro uridine and 2′-Fluoro adenosines, flanked by a resistant moiety of four 2′-*O*-Methyl uridine at each side (see Table S1†). Fig. 3a shows the profile of the three oligonucleotide candidates using cells in suspension as targeted bacteria preparation. Notably, all three specific sequences were successfully degraded by *S. pneumoniae* associated membrane-bound nucleases. The oligonucleotide P3 has shown slightly better results and has been selected as the lead probe to proceed with the specificity studies. To validate the utility of the lead P3 probe in the clinic, we have tested the probe with two isolates obtained from clinical samples (CS). Importantly, the lead P3 probes was successfully digested by bacterial samples from the clinical isolates, demonstrating the potential for detecting *S. pneumoniae* in samples from patients (Fig. 3b). Next, we performed the specificity studies by testing clinical isolates of *Streptococcus agalactiae* and *Streptococcus pyogenes*, along with other ATCC strains such as *Enterococcus faecium*, *Enterococcus faecalis* and *Staphylococcus aureus*. Fig. 3b shows the limited degradation of the lead P3 probe in presence of all non-specific bacteria cultures, demonstrating the specific targeting power of this tailored probe for *S. pneumoniae*. Additionally, we conducted tests involving three critical control bacteria, namely *S. epidermidis, E. coli*, and *K. pneumoniae*. The results revealed no interference, as the signals for *S. pneumoniae* remained clearly discriminated (Fig. S1†). Altogether, these findings highlight the usefulness of the specific nuclease activity associated with *S. pneumoniae* as a potential biomarker. They also suggest the feasibility of developing detection systems based on this catalytic feature.

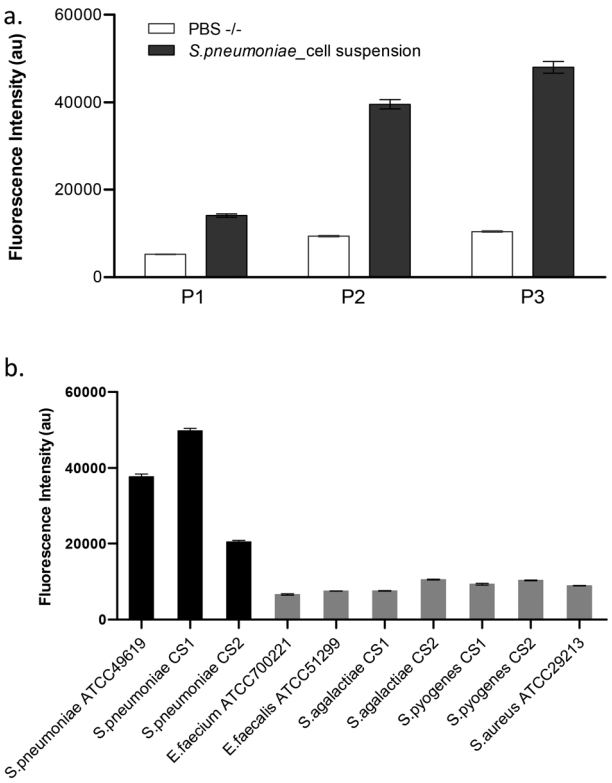


**Fig. 3** Design and assessment of a potential fluorescent probe for targeting *S. pneumoniae*. (a) Assessment of three candidate probes designed to detect *S. pneumoniae* in cell suspension. (b) Determination of the specificity of the fluorescent probe P3 for *S. pneumoniae* as the intended target bacteria, along with various non-targeted bacteria as controls. All the experiments were carried out by triplicates (mean; bars ± SD; error bars).

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### Electrochemical biosensor utilizing specific nuclease activity from *S. pneumoniae*

Once a specific oligonucleotide probe capable of detecting bacterial nuclease activity is identified, it can be integrated into various detection systems or transduction mechanisms. To demonstrate the versatility of oligonucleotide probes, we integrated the lead P3 probe into an electrochemical sensor. In this integration, the fluorescence-based system's fluorophore and quencher were replaced with an electrochemical reporter group, ferrocene (Fc), at one end, and a coupling group such as a thiol, at the other end, allowing the immobilization onto gold electrodes. To assemble the biosensor, we initiated the immobilization of the 5′-Fc-lead P3 probe using the thiol group at the 3′-end, following the description outlined in the methods section. The electrochemical detection is based on the redox properties of Fc, depending on its proximity to the electrode surface. Successful immobilization was confirmed by comparing the cyclic voltammetry (CV) profiles of the bare gold electrode (Fig. 4, green scan), exhibiting a peak of 0.39 μA at a scan rate of mV $s^{-1}$, with the electrode after immobilization of the Fc-lead P3 probe (Fig. 4, orange scan), showing a higher peak of 2.88 μA due to oxidation of Fc to $Fc^{+}$. These CV scans validate the successful immobilization of the Fc-lead P3 probe on the gold surface. Subsequently, the modified electrode was exposed to incubation with *S. pneumoniae* cells suspension preparation from bacterial cultures (Fig. 4, blue scan) for 15 minutes. We observed a reduced peak of 1.56 μA, indicating significant release of Fc from the electrode surface into the solution. This release is attributed to the nuclease activity associated with *S. pneumoniae*, which degrades the oligonucleotides and causes detachment of the fragment containing Fc. These results confirm the measurement of nuclease activity associated with a specific bacterium using an electrochemical system. To the best of our knowledge, this is the first report of an oligonucleotide utilizing an electrochemical system for the detection of nuclease activity associated with a specific bacteria.

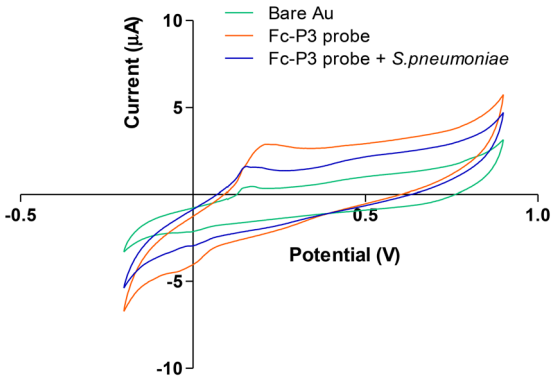


**Fig. 4** Biosensor for detecting *S. pneumoniae* using the electrochemical P3 probe. Cyclic Voltammograms scans of gold bare electrode (green) and following immobilization of the Fc-P3 probe (orange), along with the electrode response post-incubation with *S. pneumoniae* (blue).

Furthermore, the electrochemical biosensor was evaluated using Square Wave Voltammetry (SWV), another electrochemical technique that uses combined square wave and staircase potential applied to a stationary electrode, having interesting advantages such as high speed, and increased analytical sensitivity. Fig. 5a illustrates the signal obtained from the bare gold electrode (Fig. 5a, green scan) and the enhanced response following the immobilization of the ferrocene-lead P3 probe (Fig. 5a, orange scan).

Upon incubation with *S. pneumoniae* (cells in suspension) at different time points: 5, 15 and 60 min, a time-dependent reduction in current inversely proportional to the detachment of ferrocene from the oligonucleotide was observed (Fig. 5a, blue, light blue and magenta, respectively). This detachment increased the distance between ferrocene and the gold surface, resulting in decreased current. These findings are consistent with the CV results discussed earlier. These results demonstrate the feasibility and effectiveness of using an electrochemical biosensor for detecting nuclease activity. To confirm the specificity of the observed SWV current changes for *S. pneumoniae*, we conducted tests using a control bacteria (*S. aureus*) with the electrochemical biosensor. As shown in Fig. 5b, there was no notable alteration observed during the

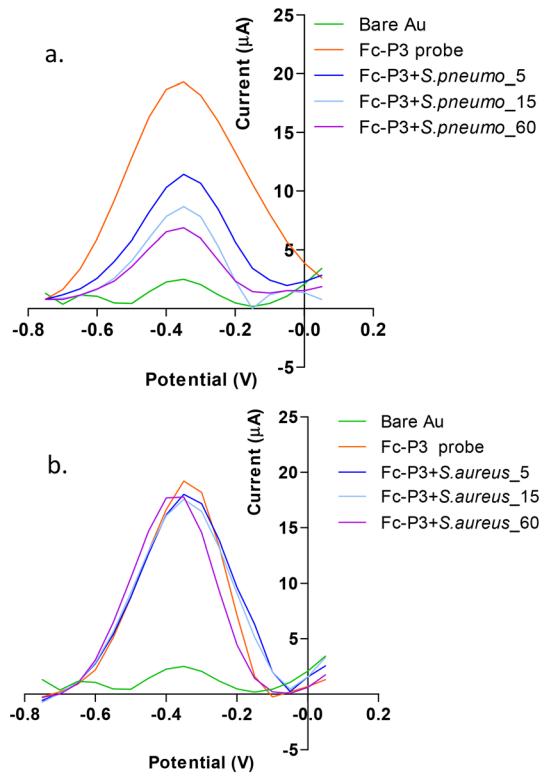


**Fig. 5** Performance of the Electrochemical P3 probe biosensor for detecting *S. pneumoniae*. (a) Square wave voltammetry (SWV) response of the electrochemical Fc-P3 probe at different time points during the incubation of *S. pneumoniae*, targeting the specific bacteria. (b) SWV responses of the Fc-P3 probe throughout the incubation of *S. aureus* at various time points, as non-targeted control bacteria.


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incubation of the control bacteria at all the measured time points. This observation indicates that the current change is indeed specific to *S. pneumoniae*.

To complete the specificity studies, we assessed the electrochemical biosensor using clinical isolates of *S. pneumoniae*, clinical isolates of non-targeted bacteria, and other ATCC strains. Fig. 6 demonstrates a significant change in current for all *S. pneumoniae* strains, including the ATCC strain and two clinical isolates, with similar electrochemical signals. In contrast, no significant change in current was observed for non-targeted bacteria, providing clear evidence of the high specificity achieved by the electrochemical biosensor. The clinical samples were verified using the standard method VITEK 2 automated microbial identification system (Table S2†). Overall, these findings highlight the successful application of an electrochemical biosensor for detecting nuclease activity associated with *S. pneumoniae*. This opens new possibilities for targeting bacteria by using electrochemical readouts and nuclease activity as biomarker. Subsequently, we assessed the sensitivity of both biosensors through a titration ranging from $10^1$ to $10^8$ CFU mL$^{-1}$ of *S. pneumoniae cultures*, as illustrated in Fig. 7. The electrochemical biosensor exhibited superior sensitivity, achieving a detection limit of $10^2$ CFU mL$^{-1}$ (Fig. 7a), surpassing the fluorescence biosensor's detection capabilities, effective from $10^6$ CFU mL$^{-1}$ and beyond (Fig. 7b). These results can be attributed to the heightened sensitivity inherent in electrochemical systems, outperforming fluorescence methods. Additionally, the catalytic features of nucleases further enhanced the performance of electrochemical sensors. Altogether, these findings underscore the dynamic detection range of both biosensors and demonstrate that the electrochemical sensor has exhibited ultra-sensitive detection capabilities. To verify the reproducibility of both methods, we tested three independent electrodes and three fluorescence probe measurements, and no significant variation was observed (Fig. 7, error bars).

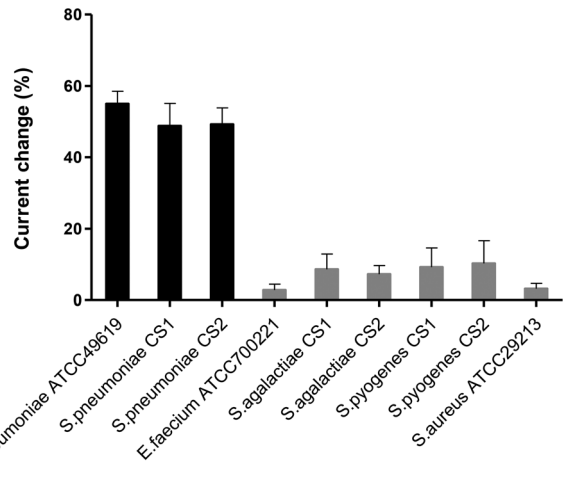


**Fig. 6** Specificity Studies of the Electrochemical Fc-P3 probes. Assessment of the electrochemical Fc-P3 probe's specificity towards *S. pneumoniae*, the targeted bacteria, as well as its evaluation against several non-targeted bacteria serving as controls. All the experiments were carried out by triplicates (mean; bars ± SD; error bars).

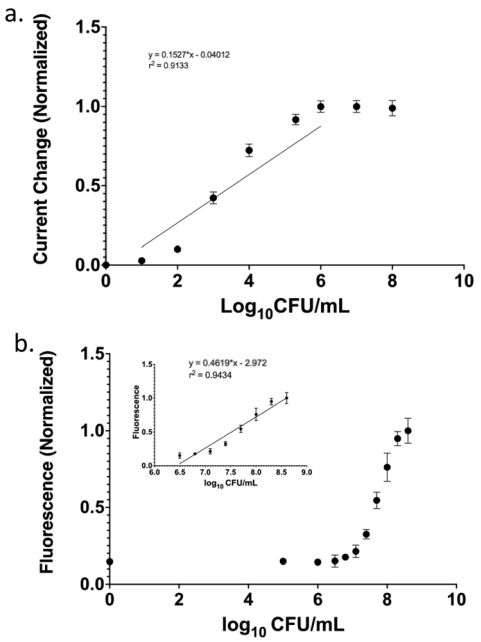


**Fig. 7** Sensitivity of electrochemical and fluorescence biosensors through titration from $10^1$ to $10^8$ CFU mL$^{-1}$ of *S. pneumoniae* cultures. (a) The electrochemical biosensor exhibits a detection limit of $10^2$ CFU mL$^{-1}$, while (b) the fluorescence sensor has a detection limit of $10^6$ CFU mL$^{-1}$. All the experiments were carried out by triplicates (mean; dots ± SD; error bars), the detection limit was calculated by simple linear regression (line).

# Experimental

### Sample preparation

All bacterial strains were propagated on tryptone soy agar with 5% defibrinated sheep blood (Thermo Scientific, Inc., Waltham, MA, USA) and the plates were incubated overnight at 37 °C. For *S. pneumoniae* strains, an airtight incubation jar containing $CO_2$ pouches (CampyGen™ Atmosphere Generation Systems) was used to support the ideal bacterial growth. The next day, 0.5 Mc Farland bacteria suspensions were prepared in Tryptic Soy Broth (TSB) (Thermo Scientific, Inc., Waltham, MA, US) and 1 mL from each suspension was transferred to a flask containing 24 mL sterile TSB. Then, the flasks were incubated at 37 °C for 12 hours. $CO_2$ pouches were also used for *S. pneumoniae* broth cultures as described before. After the incubation period, 1 mL of bacterial culture from each flask was carefully transferred into 1.5 mL Eppendorf tubes and centrifuged at 13 000 rpm for 20 minutes. Initially, to localize the nuclease activity of *S. pneumoniae*, the culture supernatants and the pellets with bacterial cells were both tested. For this purpose, culture supernatants were carefully separated after the centrifugation and the pellets were washed twice in 1 mL phosphate-buffered saline not containing any

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ions (PBS–/–) with centrifugation at 13 000 rpm for 10 minutes. The final pellets were resuspended in 100 μL of PBS (–/–) to be used as cells in suspension samples. Cells in suspension samples were also prepared for all the control bacteria along with *S. pneumoniae* and used for specificity studies.

### Screening methodology

The screening methodology was conducted in accordance with our lab's established protocols.[29,30,32] Briefly, *S. pneumoniae* supernatants and suspended cells were exposed to an oligonucleotide library comprising DNA and RNA. Subsequently, upon the identification of RNase activity as a potential biomarker, chimeric RNA libraries incorporating 2′-Fluoro and 2′-*O*-Methyl modifications were assessed using the same experimental conditions as the initial DNA and RNA libraries.[32]

### Nuclease activity assays (fluorescence detection)

An oligonucleotide library comprising of DNA and RNA-based sequences modified with a fluorophore and quencher was used to detect and differentiate nuclease activity by fluorescence. This was performed following a two-step screening method. The first screening included oligonucleotides with natural (non-modified) sequences of DNA and RNA. The second screening included chimeric probes comprising of chemically modified nucleosides, specifically 2′-*O*-Methyl or 2′-Fluoro. The sequences of the probes are provided in Table S1.† The reaction mixture for each oligonucleotide probe were prepared in Eppendorf tubes as follows: 8 μL sample + 1 μL ultrapure water + 1 μL oligonucleotide probe. The reaction mixtures were incubated at 37 °C for 1 hour. The reaction was then stopped by adding 295 μL of 10 mM EDTA prepared using PBS (–/–). From each reaction tube, 95 μL was pipetted in triplicate into a black bottom 96-well plate (Thermo Scientific, Inc., Waltham, MA, USA). The fluorescence intensity was read on a Synergy HTX Multimode Reader (Biotek Instruments Inc., USA).

### Electrochemical sensor assembly

A standard three-electrode electrochemical cell was employed for conducting the electrochemical experiments, under the monitoring of a computer-controlled Dropsens i-Stat 400 potentiostat using Dropsens 8400 software (version 3.6 20B0514). The working electrode consisted of gold, while the auxiliary electrode was crafted from platinum wire. The silver reference electrode served as the point of reference for all potential measurements. The gold electrode was prepared by treating it with a minimal volume of piranha solution (3 : 1 $H_2SO_4$ (95%) : $H_2O_2$ (33%)) for a duration of 5 minutes to remove organic residue from the gold surface. It was then washed with ethanol and water, followed by polishing on a polishing cloth using alumina slurries. The thiol-modified Fc-P3 Probe (1 μM) were combined with a 3 mM disulfide bond reducing agent known as tris(2-formyl ethyl) phosphine hydrochloride (TCEP) in PBS (100 mM, pH 7.4, 1 mM $MgCl_2$). This mixture was incubated for 15 minutes at 37 °C before being applied onto the electrode surface. Subsequently, 5 μL of the probe-TCEP solution was carefully placed onto the working electrode and left for a period of 16 hours at 25 °C. Following this, the electrode surface underwent three rounds of washing, each with 50 mL of PBS. The Fc-P3 probe-modified gold electrodes were immersed in a 1× PBS buffer solution containing 100 mM KCl.

### Cyclic voltammetry (CV)

CV measurements were performed within the range of −0.2 V to 0.9 V, employing a scan rate of 50 mV $s^{-1}$. All electrochemical measurements were performed at a temperature of 25 °C. For each experimental iteration, 25 measurements were recorded, and the average values were reported.

### Square wave voltammetry (SWV)

SWV scan were acquired at a frequency of 60 Hz and an amplitude of 50 mV for the Fc-P3 probe modified electrodes. The SWV signals were monitored over time after incubation with the targeted bacteria (*S. pneumoniae*) or control bacteria, and the resulting signal gain was expressed as the relative change in SWV peak current compared to the initial peak current (% signal change). For each experimental iteration, 25 measurements were recorded, and the average values were reported.

### Clinical sample verification method

All clinical strains were isolated in Prof. Dr A. Ilhan Özdemir State Hospital medical microbiology laboratory (Giresun, Turkey) and identified using VITEK 2 automated microbial identification system (BioMerieux, France).

### Sensitivity

The electrochemical and fluorescence detection of bacteria was conducted by acquiring signals over a gradually increasing concentration range from $10^1$ to $10^8$ CFU $mL^{-1}$, as previously described.[33] The detection limit was calculated using simple linear regression analysis with Graph Prism.

## Conclusions

This study introduces an oligonucleotide probe designed for the specific targeting of *S. pneumoniae*. Through screening experiments, a chimeric oligonucleotide containing 2′-*O*-Methyl as a protective moiety, along with 2′-Fluoro uridines and adenosines, was identified as the optimal specific moiety for targeting *S. pneumoniae* (P3 probe). This probe functions as a substrate that targets the nucleases associated with the membrane-bound structure of *S. pneumoniae*, utilizing this specific nuclease activity as a biomarker.

The design and validation of P3 probe have demonstrated significant degradation by *S. pneumoniae* and minimal degradation by non-specific bacteria. Notably, the incorporation of the lead P3 probe into an electrochemical biosensor showcased its efficacy in detecting nuclease activity. This represents the first instance of an electrochemical sensor being employed for the detection of nuclease activity as a biomarker to identify

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specific bacteria. Both sensors demonstrated successful differentiation of *S. pneumoniae* from non-specific bacteria, showcasing remarkable detection capabilities. The electrochemical detection method achieved an impressive level of sensitivity, detecting as low as $10^2$ CFU $mL^{-1}$. In contrast, the fluorescence sensor exhibited a more moderate detection limit, effective from $10^6$ CFU $mL^{-1}$. These findings underscore the efficacy of both sensors in specifically identifying *S. pneumoniae*, with the electrochemical approach standing out for its ultra-sensitive detection capabilities.

These findings highlight the potential of nuclease activity as a dependable biomarker for targeted bacterial detection, and they highlight the versatility and promise of electrochemical biosensors in this context.

## Author contributions

Garazi Goikoetxea: investigation, methodology, visualization, formal analysis, writing – review & editing. Khadija-Tul Kubra Akhtar: investigation, formal analysis, writing – review & editing. Alona Prysiazhniuka: investigation, formal analysis, writing – review & editing. Baris A. Borsa: investigation, formal analysis, writing – review & editing. Mehmet Ersoy Aldag: investigation, formal analysis, writing – review & editing, resources. Murat Kavruk: investigation, formal analysis, writing – review & editing. Veli C. Ozalp: investigation, validation, supervision, resources, formal analysis, writing – review & editing. Frank J. Hernandez: writing – original draft, supervision, project administration, methodology, funding acquisition, formal analysis, conceptualization.

## Conflicts of interest

There are no conflicts to declare.

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