Received: 12 April 2023 | Revised: 9 September 2023 | Accepted: 11 September 2023

DOI: 10.1002/elan.202300119

RESEARCH ARTICLE

ELECTROANALYSIS

Non-invasive detection of Crohn's Disease with label-free detection of calprotectin by impedimetric aptasensor

Çiğdem Gözde Aslan[1, 2] | **Hilmiye Deniz Ertugrul Uygun**[3] | **Zihni Onur Uygun**[1, 4] | **Nalan Gülşen Ünal**[5] | **Yasemin Akcay**[1]

[1]Ege University, Faculty of Medicine, Department of Medical Biochemistry, İzmir, Türkiye

[2]Biruni University, Faculty of Medicine, Department of Medical Biochemistry, İstanbul, Türkiye

[3]Dokuz Eylül University, Center for Fabrication and Application of Electronic Materials, İzmir, Türkiye

[4]Kafkas University, Faculty of Medicine, Department of Medical Biochemistry, Kars, Türkiye

[5]Ege University, Faculty of Medicine, Department of Gastroenterology, İzmir, Türkiye

Correspondence
Zihni Onur Uygun, Ege University, Faculty of Medicine, Department of Medical Biochemistry, İzmir, Türkiye.
Email: onur.uygun@kafkas.edu.tr

Funding information
Ege University Scientific Projects Coordination, Grant/Award Number: TGA-2019-21017

Abstract

Crohn's disease, a chronic inflammatory disorder affecting the gastrointestinal tract, is on the rise, posing significant health challenges due to its potential long-term impact on intestinal health. Early and accurate detection of this disease is crucial for effective treatment. This study introduces an innovative approach to diagnosing Crohn's disease by utilizing a rapid and precise biosensor system as an alternative to conventional detection techniques. Utilization of an S100 A8 targeting aptamer for the specific identification of calprotectin, a well-established biomarker associated with Crohn's disease was used as a biorecognition receptor. The aptamer is immobilized on gold nanoparticles via cysteamine and avidin to construct a biosensor. The biosensor was compared with traditional quantitative lateral-flow tests (QLFA). The developed biosensor demonstrates that detection in 900 seconds. Performance parameters, including a limit of detection (LOD) as 5.57 μg/g and a limit of quantification (LOQ) as 16.89 μg/g, are determined through electrochemical impedance spectroscopy (EIS). Accordingly, the calibration curve equation was calculated as $y = 3.9736x + 1570.4$ and the regression coefficient was found as $R^2 = 0.9917$. These results showed that biosensor's capability to reliably measure calprotectin levels in real samples.

KEYWORDS
biosensor, calprotectin, chronoimpedance, Crohn's disease, impedance

1 | INTRODUCTION

Inflammatory bowel diseases, commonly referred to as IBDs, encompass a collection of chronic inflammatory disorders that primarily affect the gastrointestinal system [1]. These diseases, including Crohn's disease (CD) and ulcerative colitis (UC), are characterized by periods of inflammation and periods of remission [2]. This chronic and recurrent inflammation can lead to various symptoms and complications both within the gastrointestinal tract and in other parts of the body. such as joints, skin, and eyes [3]. Crohn's disease is a chronic inflammatory disorder primarily affecting the gastrointestinal tract. It is characterized by periods of exacerbation and remission, leading to a range of symptoms that can significantly impact a patient's quality of life. Early and accurate diagnosis of Crohn's disease is crucial for effective management and improved patient

outcomes. Diagnosing Crohn's disease involves a combination of clinical assessment, laboratory tests, imaging studies, and endoscopic procedures [4]. Physicians evaluate the patient's medical history, including symptoms such as abdominal pain, diarrhea, weight loss, fatigue, and nutritional deficiencies. In order to assess inflammation and rule out other potential causes of symptoms. The biomarkers can help to diagnose that disease such as C-reactive protein (CRP), which is an acute-phase reactant produced by the liver in response to inflammation [1]. Elevated CRP levels are commonly observed in CD patients during active disease phases. However, that biomarker is not specific for the CD. Another clinical test is Erythrocyte Sedimentation Rate (ESR) measures the rate at which red blood cells settle in a test tube over a specified time period. Like CRP, elevated ESR is indicative of inflammation and can provide additional information about any disease activity. Anti-Saccharomyces cerevisiae Antibodies (ASCA) are antibodies that target a yeast commonly found in the intestines. Their presence in the blood has been associated with CD and may aid in distinguishing CD from other forms of IBD. Anti-Neutrophil Cytoplasmic Antibodies [5] (ANCA) while more commonly associated with ulcerative colitis, ANCA can also be present in CD patients with colonic involvement. Their detection can provide additional diagnostic and prognostic information. Proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and IL-12 are often elevated in CD [6]. These molecules play a central role in driving inflammation and are targeted by many CD therapies. Among all of the biomarkers for Crohn's disease, Fecal Calprotectin is more specific to diagnose CD. Calprotectin is a calcium-binding protein released by neutrophils during inflammation [7]. Calprotectin, in other words, is a dimer S100A8/A9 protein family of S100. Its levels increase in response to inflammation in the gastrointestinal tract, making it a sensitive indicator of mucosal inflammation. In CD, ongoing inflammation leads to elevated levels of calprotectin in the stool [1]. Fecal calprotectin provides a non-invasive method for assessing intestinal inflammation [8]. Unlike invasive procedures like endoscopy, which require direct visualization of the gastrointestinal tract, calprotectin measurement offers valuable information without requiring a tissue biopsy. Monitoring calprotectin levels allows clinicians to gauge the severity of intestinal inflammation. Elevated calprotectin levels often correlate with active disease, and a decrease in levels indicates a response to treatment and disease remission. In conclusion, sensitive and selective detection the fecal calprotectin is important for the disease assessment and the diagnosis. The most employed method to detect calprotectin is ELISA [6] a widely used technique for quantifying biomolecules and Lateral Flow Immunoassay (LFIA) [9] also known as rapid or point-of-care testing, offers a simpler and quicker way to measure fecal calprotectin levels. Although, LFIA is used as a routine hospital analysis, it has limitations such as semi-quantitative detection, selectivity and sensitivity. Biosensors are useful mini analyzers, which provide low-cost and sensitive detection, with the combination of biorecognition receptor and a transducer [10]. Among of the different biorecogtinition receptors, aptamers are unique and synthetic single stranded DNAs can be designed for any targets and their binding constants can be arranged and useful for wide range targets [11–13]. Therefore, we designed a new biosensor to detect calprotectin with different biorecognition receptor for A8 protein and used in the stool samples. This label-free detection technique was designed by using electrochemical impedance spectroscopy (EIS) a sensitive and capable of detect electrode electrolyte interface investigation technique [14]. A gold nanoparticle electrode was engineered by cysteamine [15], avidin and biotin-modified aptamers as calprotectin biosensor. This biosensor was used for label-free detection of the calprotectin by using electrochemical impedance spectroscopy (EIS).

2 | MATERIALS AND METHOD

2.1 | Materials and equipment

The research was carried out with permission of Ege University Research Ethics Committee with the permission number of 10–10.2T/33. All chemicals were obtained from Merck (USA). Potentiostate, Palmsens 3, and its interface were obtained from Palmsens B.V. (Netherlands). Gold nanoparticle electrodes were obtained from Dropsens (110GNP, Dropsens, Spain). Quantum Blue® fCALextended Quantitative Lateral Flow Assay was obtained from the Bühlmann Laboratories (AGBaselstrasse 554124 Schönenbuch, Sweden).

2.2 | Methods

2.2.1 | Stool sample collection and analysis

Stool samples were collected from the CD diagnosed patients, weighted and extracted, after the extraction process the extracts were stored in a freezer at −80 °C until use. The extraction process is as follows; approximately 1 g of feces was diluted with 4 ml of extraction buffer and thoroughly vortexed. The diluted samples were then

incubated at 18–28 °C for at least 5 min. 50 μL of this extract was taken and 450 μL of extraction buffer was added (1:10 dilution), vortexed again and centrifuged at 3000 g for 5 min, then the supernatant was separated [9], divided in two samples for biosensor and LFA analysis and stored at −80 °C as extract until measurement. The sample analysis was carried out by Quantum Blue® fCA-Lextended Quantitative Lateral Flow Assay (BUHLMANN Diagnostics Corp, USA).

2.2.2 | Biosensor modifications and optimizations

Biosensor characterizations were observed by electrochemical and physicochemical techniques. Electrochemical techniques were cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). EIS was also used for the quantification of the calprotectin. CV and EIS was performed in 5 mM $Fe(CN)_6^{3-/4-}$ redox probe couple and 100 mM KCl in pH = 7 50 mM phosphate buffer. EIS curves were calculated by fitting the impedance data on the elemental circuit model. In this circuit model, R1 represents the redox probe solution impedance, R2 represents the biosensor surface electron transfer resistance, which is used to measure calprotectin signals, C is the capacitive charge of the surface and W is the mass transfer resistance (Warburg impedance) [16]. In this study, cyclic voltammetry and EIS were used to determine the electrode surface characteristics. EIS was also used as the main analysis methods for the electrochemical detection of Calprotectin. EIS and CV were performed in a redox probe solution and parameters were optimized in previous methods, and EIS results were calculated using a specific circuit model. As a biorecognition receptor for the detection of calprotectin, the aptamer sequence for calprotectin was selected [17]. The biosensor modification begun with the modification of the gold nanoparticle electrodes (GNPE) washed with distilled water and dried under a nitrogen gas flow. The GNPE was then immersed in 100 mM cysteamine (Cys) for 1 h to form gold nanoparticle-cysteamine bonds via S groups of cysteamine. Then, the amino groups on the electrode (GNPE-Cys) were activated by dropping 20 uL, 5% glutaraldehyde on the modified electrode for 30 min. Subsequently, 1 mg/mL pH = 7.4 avidin solution was dropped onto the activated GNPE-Cys and incubated for 1 h at room temperature [5]. Thus, the binding of biotin-modified aptamers to the surface via high affinity between avidin-biotin interaction ($Km = 1.3\times10^{-15}$ M) were facilitated by using aptamers. After the electrode was washed to remove unbounded aptamers, it was dipped in a solution containing 1 mg/mL human serum albumin (HSA) to block the free activated residues of Cys. Those modification steps were tested by CV, EIS and AFM. Aptamer (biorecognition receptor) and calprotectin binding time optimization was carried out by chronoimpedimetric detection, which was performed by assessing GNPE/Cys/Avidin-Aptamer for calprotectin analysis time in PBS to monitor impedance increase on the surface the function of the time. Calprotectin detection was monitored at 180 mV frequency 330 Hz. These parameters were obtained from the Bode plot of the Aptamer modified and Calprotectin bonded EIS results of the biosensor. Afterward, the biosensor was tested for the calibration curve, LOD and LOQ. LOD and LOQ values were calculated considering the standard deviation (SD) of repeated measurements of the lowest concentration and the slope of the calibration curve (m) (LOD = 3.3(SD/m)), LOQ = 10(SD/m)). For reproducibility, calibration curve tests were repeated ten times to assess the regression coefficients. Reproducibility measurements were performed by adding the standard of the target biomolecule to the sample and measuring this sample at least three times to determine the recovery value. Real sample analysis (regression) analysis was carried out by comparing the biosensor with LFA [18].

3 | RESULTS AND DISCUSSION

In electrochemical processes, electron transfer from the electrode to the solution (or vice versa) is critical. This transfer of electrons is necessary for redox reactions to occur. However, when biomolecules are immobilized on the electrode surface, they can act as insulating barriers, hindering the flow of electrons those released from the electrochemical transformation of the redox probe. In brief, GNPE was modified by cysteamine, activated with glutaraldehyde, avidin, and then biotin-labeled aptamer, simultaneously. Investigation of the biosensor surfaces were performed layer-by-layer with CV and EIS (Figure 1A and B). Cys layer in pH = 7 redox probe solution has positive charges. Those charges attracted the negatively charged redox probe. That electrostatic interaction and the diffusion towards surface of the redox probe movement increase the current. Redox probes are molecules that can undergo reversible oxidation-reduction (redox) reactions (Figure 1A) and decreased the impedance (Figure 1B) because of the more redox probe released electrons. That mass transfer resistance (Warburg impedance) became dominant against electron transfer resistance. The electrons increased the current and decreased the impedance. That situation explains that Cys modification was successful. Afterwards the Avidin

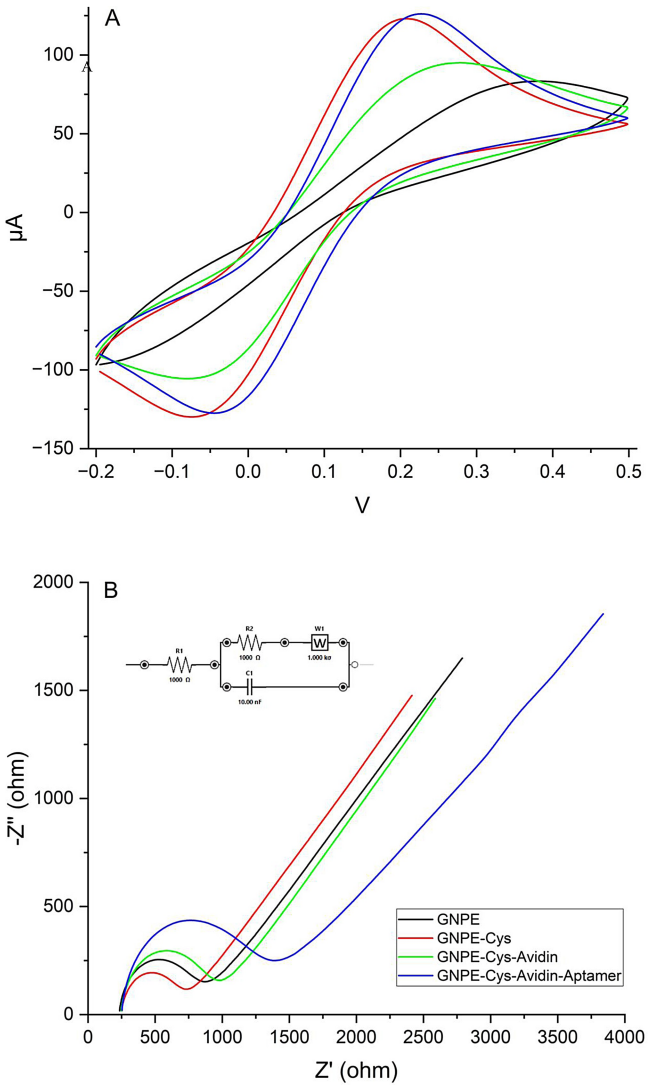

FIGURE 1 A; CV curves show the immobilization procedures of the GNPE-Cys-Avidin-Aptamer-S100A8-9 protein modification, B; EIS data shows the modifications of the GNPE-Cys-Avidin-Aptamer-S100A8-9 protein modification (Red; GNPE, Green; GNPE-Cys, Blue; GNPE-Cys-Avidin, Yellow GNPE-Cys-Avidin-Aptamer).

immobilization and the Aptamer immobilization formed an insulating layer. Those layers prevent the redox probe to electrode surface and the redox probe released the electrons on tha layer of avidin-aptamer layer. That layer causes a resistance against the redox probe electrons. Moreover, aptamer layer has phosphate backbone, which is negatively charged, that repels the redox probe from the surface by decreasing CV current peak and increasing those immobilizations were decreased the CV peaks and increased the EIS. The surface showed resistivity against the released electrons from redox probe. Impedance spectrums are calculated by using a proper equivalent circuit model. That circuit model is characterized by the obtained impedance curve that showed in figure 1B. R1 and R2 represents the impedance of the solution and biosensor surface resistances, respectively. Warburg (W) impedance represents the redox probe movement to surface and C1 is the capacitive double layer of the biosensor [19]. Those modifications were also observed by an atomic force microscopy (Figure 2). As can be seen in the figure, the surface modifications change the surface topographical structure. This method was used to verify electrochemical analyses.

It is the most time-consuming optimization step is the detection time for the afinity based biosensor with label-free detection. In this study we used chronoimpedimetric detection by dipping electrode in buffer as blank and 10 μg/g standard calprotectin, which is capable of detect non-faradaic impedance changes on electrode surface. In order to observe the frequency of the chronoimpedimetric detection we used Bode-Plot of the calprotectin-bonded EIS (S1). The frequency has been chosen at the point, where the phase angle steady and the impedance shows increase characteristics. Therefore, frequency and potential were determined as 180 mV and 330 Hz frequency (S1). The binding time of the target protein, calprotectin, to the aptamer was determined by chronoimpedance and showed in Figure 3 that GNPE-Cys-Avidin-Aptamer shown in blue as blank and calprotectin binding in red chronoimpedance curves. As for the measurement time, it was determined as 900 seconds (detection time) that point separates the meaningful signal from the PBS.

After the optimization of Calprotectin detection time, calibration curve was constructed with faradaic EIS in redox probe. The standards were dropped on the biosensor and incubated for 900 seconds and impedance was performed in a redox probe. Calibration curve was prepared by using EIS shown in Figures 4A and plotted EIS data against calibrations in 4B. In Figure 4A, impedance data were obtained and calculated according to Figure 4 data that fitted on the circuit model of Randles that mentioned previously, and R2 values were calculated in Figure 4B against concentrations. Accordingly, the calibration curve equation was calculated as $y=3.9736x+1570.4$ and the regression coefficient was found as $R^2=0.9917$. The LOD, and LOQ were found as 5.57 μg/g and 16.89 μg/g. Then, the biosensor was compared with real samples with the Quantum Blue® fCALextended Quantitative Lateral Flow Assay (LFA), which is a lateral flow technology (using a highly specific monoclonal antibody to calprotectin), with full quantification by a reading device that can measure S100A8-9 protein levels. 30 samples of extracted stools were measured using this method and biosensor. The samples were dropped on the biosensor and incubated for 900 seconds and impedance was

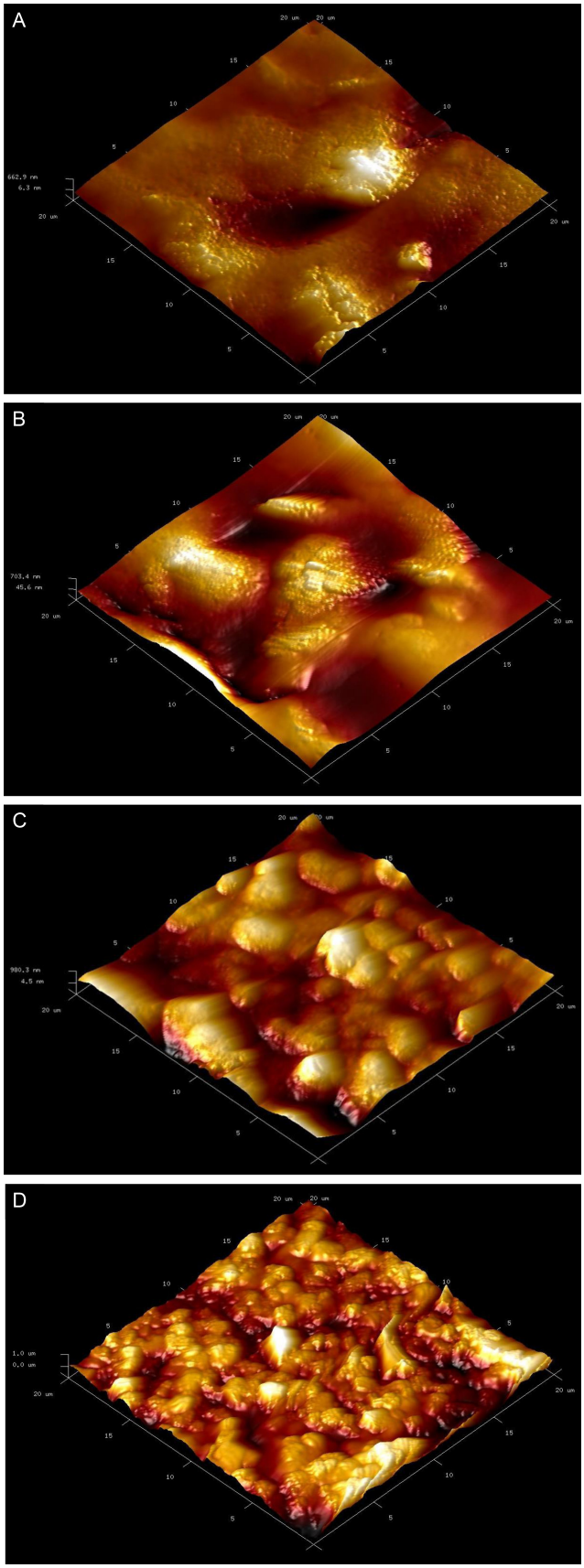

FIGURE 2 Atomic force microscopy pictures of the step-by-step electrode modifications A: GNPE, B: GNPE/Cys, C: GNPE/Cys/Avidin, D: GNPE/Cys/Avidin/Aptamer.

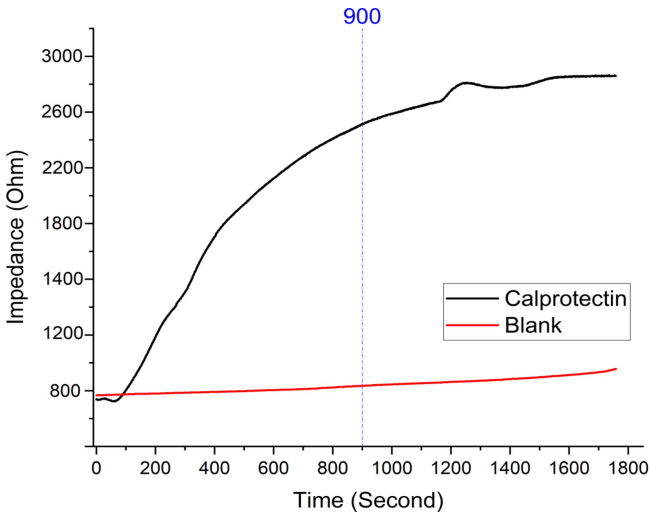

FIGURE 3 Chronoimpedimetric monitoring of S100A8-9 binding to the biosensor, red is baseline, black is S100A8-9 chronoimpedance signal increases.

performed in a redox probe. The measurement results are given as regression in Figure 5.

The selectivity study was carried out by testing artificial samples containing S100A8 and S100A9 separately with the biosensor. A column graph was prepared based on the EIS measurements shown in Figure 6A and percentages were calculated in Figure 6B. The response of the biosensor to the S100A8-9 protein was accepted as % 100 and the response of the biosensor to the S100A8 and S100A9 proteins was calculated relatively.

4 | CONCLUSION

In conclusion, the rising prevalence of Crohn's disease presents a formidable challenge to healthcare systems worldwide, emphasizing the critical need for accurate and early diagnostic tools. By an aptamer targeting S100 A8 and immobilizing it on gold nanoparticles, this biosensor provides a rapid and precise detection of calprotectin – a well-established biomarker linked to Crohn's disease. The extensive comparative assessments against a conventional method, lateral-flow test, shows the biosensor's exceptional accuracy and efficacy. A chronoimpedimetric test recognizes that detection of calprotectin takes just 900 seconds. The calculated calibration curve equation and regression coefficient solidify its consistency in measuring calprotectin levels. The successful integration of advanced technology with clinical diagnostics demonstrated in this study not only offers an innovative solution for Crohn's disease diagnosis but also sets a precedent for the ongoing convergence of healthcare and cutting-edge research. Moreover, this

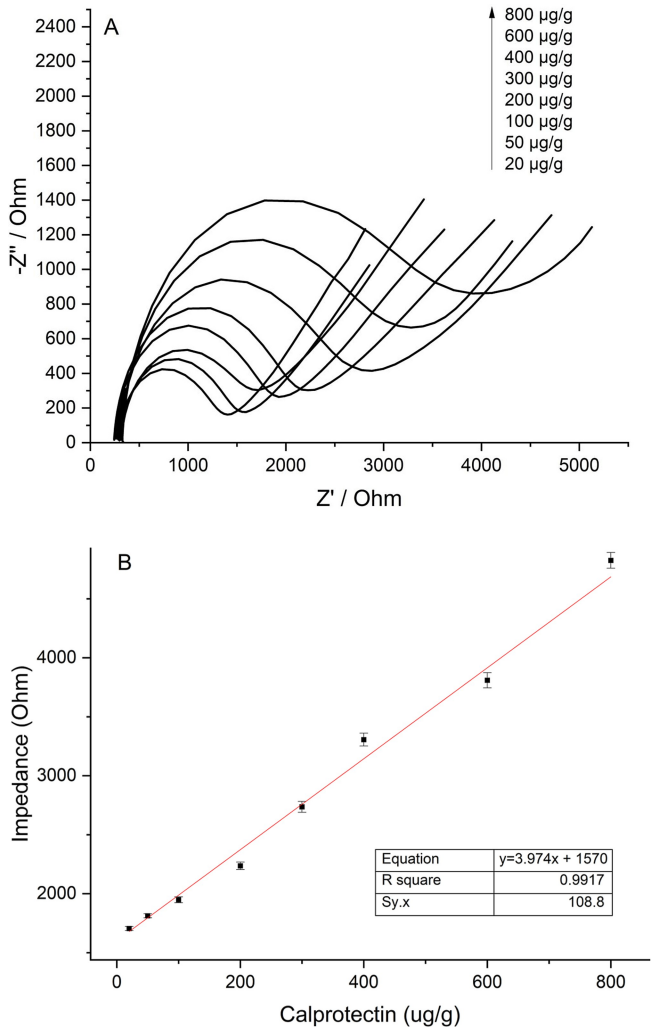

FIGURE 4 A; EIS data of the standard calprotectin concentrations between 20–800 µg/g levels. B; Calibration curve of the calprotectin by using electron transfer resistance on y-axis and S100A8-9 protein concentration on x-axis.

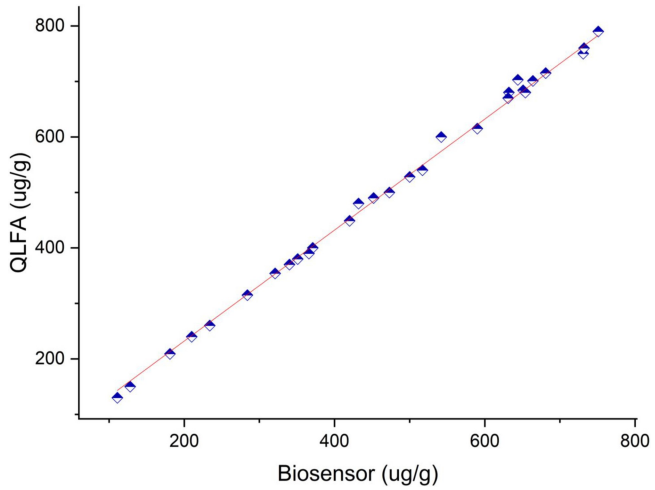

FIGURE 5 Regression plot of LFA with biosensor comparisons of the 30 stool sample extracts.

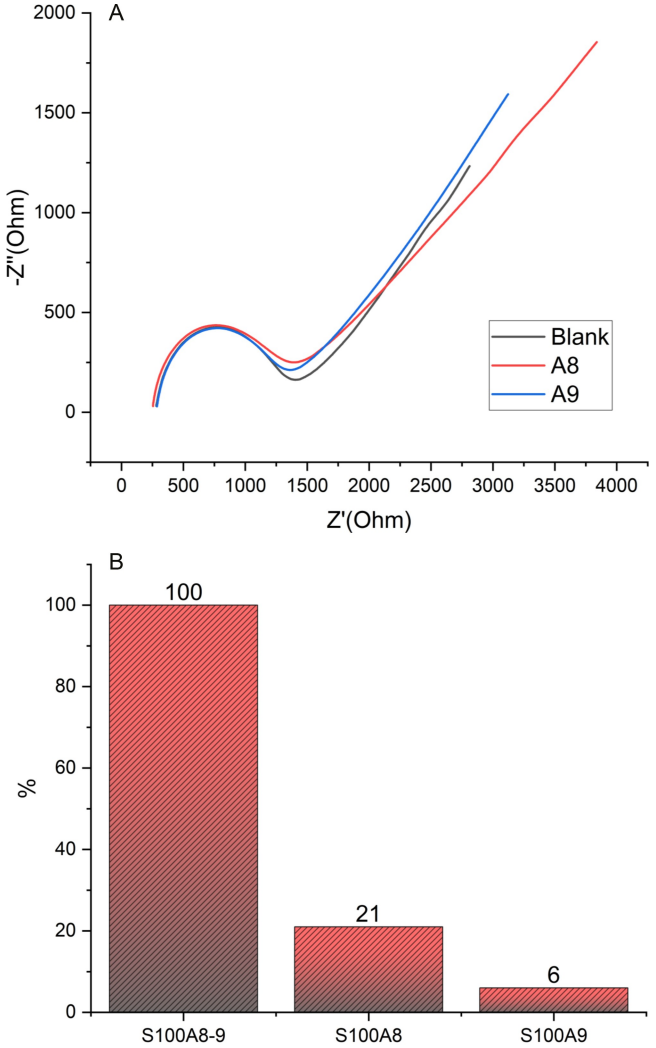

FIGURE 6 A; The selectivity studies of the GNPE-Cys-Avidin-Aptamer-biosensor (black GNPE-Cys-Avidin-Aptamer), Red and blue EIS S100A8 and S100A9, respectively. B; Percentage comparison by using EIS signals of calprotectin biosensor and S100A8 and S100A9.

biosensor holds the potential to significantly improve the quality of life for individuals grappling with Crohn's disease and contribute to the broader advancement of diagnostic methodologies in the area of gastrointestinal disorders.

AUTHORS CONTRIBUTIONS

Çiğdem Gözde ASLAN: Experimenting, Writing, Zihni Onur UYGUN: Methodology, Hilmiye Deniz ERTUĞRUL UYGUN: Conceptualization and writing, Nalan Gülşen ÜNAL: Clinical Assessment of The Samples and Collection, Yasemin AKÇAY: Revisions, Methodology and Funding acquisition.

ACKNOWLEDGEMENT

This study was supported by Ege University Scientific Projects Coordination by the consent number of TGA-2019-21017 and The Scientific and Technological Research Council of Türkiye (TÜBİTAK) Turkish Academic Network and Information Center (ULAKBIM) for OpenAccess.

CONFLICT OF INTEREST STATEMENT

Authors declare that all data can be shared and accessed by the permission of the journal publication rules.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

REFERENCES

1. J. Langhorst, S. Elsenbruch, J. Koelzer, A. Rueffer, A. Michalsen, G. J. Dobos, *Am. J. Gastroenterol.* **2008**, *103*, 162–169.
2. C. Ray, P. Sagar, *Surgery* **2017**, *35(8)*, 439–442.
3. J. Burisch, T. Jess, M. Martinato, P. L. Lakatos, *J. Crohns. Colitis* **2013**, *7*, 322–337.
4. A. Buisson, J.-B. Chevaux, G. Bommelaer, L. Peyrin-Biroulet, *Dig. Liver Dis.* **2012**, *44*, 453–460.
5. C. Roozendaal, C. G. M. Kallenberg, *Clin. Exp. Immunol.* **1999**, *116*, 206.
6. H. C. Reinecker, M. Steffen, T. Witthoeft, I. Pflueger, S. Schreiber, R. P. MacDermott, A. Raedler, *Clin. Exp. Immunol.* **2008**, *94*, 174–181.
7. D. Acevedo, M. P. Salvador, J. Girbes, N. Estan, *J. Clin. Med. Res.* **2018**, *10*, 396–404.
8. S. Wang, R. Song, Z. Wang, Z. Jing, S. Wang, J. Ma, *Front. Immunol.* **2018**, *9*, 1298.
9. J. Xing, J. Li, Z. Yan, Y. Li, X. Liu, L. He, T. Xu, C. Wang, L. Zhao, K. Jie, *J. Orthop. Surg. Res.* **2022**, *17*, 1–12.
10. Z. O. Uygun, *Fundam. Sens. Technol. Princ. Nov. Des.* **2023**, 45–62.
11. D. Chen, D. Yao, C. Xie, D. Liu, *Food Control* **2014**, *42*, 109–115.
12. Z. O. Uygun, F. G. Sağin, *Turkish J. Biochem.* **2021**, *0*, DOI 10.1515/TJB-2021-0056.
13. M. A. Tabrizi, P. Acedo, *Biosens.* **2022**, *12*, 142.
14. H. D. Ertuğrul, Z. O. Uygun, *State Art Biosens. - Gen. Asp.* **2013**, 179–196.
15. Z. O. Uygun, Ç. Şahin, M. Yılmaz, Y. Akçay, A. Akdemir, F. Sağın, *Anal. Biochem.* **2018**, *542*, 11–15.
16. Z. O. Uygun, H. D. Ertuğrul, Z. O. Uygun, *Sens. Actuators B* **2014**, *202*, 448–453.
17. L. Chen, J. Li, X. Q. Zhang, L. Song, C. Qian, J. Wen Ge, *Beijing Da Xue Xue Bao.* **2014**, *46*, 698–702.
18. Q. Delefortrie, P. Schatt, A. Grimmelprez, P. Gohy, D. Deltour, G. Collard, P. Vankerkhoven, *Clin. Biochem.* **2016**, *49*, 268–273.
19. H. D. Ertuğrul Uygun, Z. O. Uygun, *Handb. Nanobioelectrochemistry* **2023**, 919–932.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ç. G. Aslan, H. D. Ertugrul Uygun, Z. O. Uygun, N. G. Ünal, Y. Akcay, *Electroanalysis* **2023**, *35*, e202300119. https://doi.org/10.1002/elan.202300119