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Electrochemical antigenic sensor for the diagnosis of chronic Q fever

Eirini Mathioudaki [a,*], Yiannis Alifragis [a], Maria Fouskaki [a], Dimosthenis Chochlakis [b], Hao Xie [c], Anna Psaroulaki [b], Georgios Tsiotis [a], Nikolaos Chaniotakis [a]

[a] Department of Chemistry, University of Crete, GR-71003 Voutes, Greece
[b] Laboratory of Clinical Microbiology and Microbial Pathogenesis, School of Medicine, University of Crete, GR-71110 Heraklion, Greece
[c] Max Planck Institute of Biophysics, Max-von-Laue-Straße 3, D-60438 Frankfurt am Main, Germany

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ABSTRACT

In this work, we report the development of an impedimetric biosensor for the direct, quick, and easy diagnosis of chronic Q fever. The biosensor is based on highly sensitive antigens that can selectively recognize antibodies against *Coxiella burnetii*. The biosensor is based on the immobilization of antigens onto a gold electrode using the EDC/NHS immobilization methodology. The detection is performed by impedance spectroscopy that monitors specific frequencies which provide the maximum sensitivity for the biosensor. Q fever antibodies that are present in the sera of patients interact selectively with the biosensor antigens, thereby altering the impedance of the biosensor surfaceand generating a large impedance change within a few seconds. The biosensor allows for the specific serological detection of chronic Q fever, while the developed system can also be modified for the detection of other biomarkers, such as the ones against acute Q fever.

Introduction

Coxiella burnetii is an obligate intracellular bacterial pathogen with a worldwide distribution that causes Q fever infection (Angelakis and Raoult, 2010). The clinical forms have been described as acute and chronic Q fever disease. Generally, acute Q fever is mild and self-limiting, with a variety of symptoms, the common of which are fever and fatigue. This influenza-like manifestation of the disease often leads to low clinical suspicion for the detection of the pathogen since samples are analyzed several days post contact with the pathogen and after the onset of symptoms (Anderson et al., 2013). On the other hand, chronic Q fever is a life-threatening disease, with symptoms ranging from endocarditis to chronic hepatitis or chronic vascular infections (Million and Raoult, 2015). In the case of the chronic form of the disease, early and accurate diagnosis is vital for the protection of the patients' health, as it can be lethal if it remains untreated (Angelakis and Raoult, 2010). However, early diagnosis of Q fever is a challenging task, considering the limitations of the current diagnostic methodologies (Angelakis and Raoult, 2010; Million and Raoult, 2015; Fournier et al., 1998; Bacarese-Hamilton et al., 2004).

The accuracy of diagnosis of chronic Q fever delivered by the existing methodologies is very low, ranging from 33 % to 64 %, rendering these methods nonspecific for the diagnosis of the chronic form of the disease. The detection of chronic Q fever in blood or tissues can also be achieved by culturing the bacterium however, this method is only 50–60 % efficient. For these reasons, the most widely used diagnostic method for the disease is the one based on serology, since following infection, IgG antibodies are persistent in the blood for months or even years (Million and Raoult, 2015; Fournier et al., 1998).

The laboratory serological methods for Q fever diagnosis include indirect immune fluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and complement fixation (Million and Raoult, 2015; Fournier et al., 1998). Although the IFA is the current gold standard serologic assay for Q fever, all above- mentioned techniques have several limitations. The limitations include large sample/reagent volume requirements, complex protocols, and large variability in the analytical specifications among laboratories (Bacarese-Hamilton et al., 2004). In addition, all the methods are based on the antigen obtained from *C. burnetii,* which is hazardous and difficult to handle, since specialized equipment and BSL3 safety level facilities are required (Angelakis and Raoult, 2010). The inherent complexity of whole cell antigen production, and the subjective nature of the IFA lead to inconsistencies in test results.

A diagnostic assay based on recombinant proteins would overcome the above-mentioned difficulties, and it would also improve the test efficacy. Several immunoproteomic studies on *C. burnetii* have been reported based on several seroreactive proteins, but only a few of them have been validated by ELISA (Coleman et al., 2007; Sekeyová et al.,

* Corresponding author.
E-mail address: chemp927@edu.chemistry.uoc.gr (E. Mathioudaki).

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2009; Papadioti et al., 2011; Skultety et al., 2011; Xiong et al., 2012). A recent study in our laboratory resulted in the successful expansion of a subset of *C. burnetii* immunoreactive proteins, which have been validated by ELISA (Miller and Kersh, 2020; Mathioudaki et al., 2021; Psaroulaki et al., 2020; Vranakis et al., 2019). Of a series of such proteins, CBU_1910 (Com1) and CBU_1718 (GroEL) demonstrated high sensitivity and specificity to chronic Q fever diagnosis (Psaroulaki et al., 2020; Vranakis et al., 2019). Lately, numerous methods using specific antigens have been developed for the diagnosis of diseases. These methods rely on pathogen detection through optical, electrochemical, electrical and mechanical sensors (Zhao et al., 2022; Koo et al., 2019). The operation principle lays on the formation of the antigen- antibody complex that will lead to a signal change (Lisdat and Schäfer, 2008). To name just a few, immunosensors have been developed for the diagnosis of *Salmonella Typhimurium*, *Staphylococcus aureus*, various viral infections and different types of cancers (Singh et al., 2020; Zhao et al., 2022). However, the antigenic proteins of C. *burnetii* have not been used for the development of Q fever diagnostic biosensors, although there are a few reports of Q fever biosensors based on DNA detection (Koo et al., 2019; Park et al., 2021) and purified whole cell (Zhang et al., 2020).

Electrochemical Impedance Spectroscopy (EIS) is a very sensitive and accurate surface monitoring technique for the detection of antigen–antibody interaction and, thus, it can be used for the development of specific, highly accurate immunosensors. EIS – based biosensors have already found several applications as diagnostic tools (Hou et al., 2006; Chen et al., 2006; Hnaien et al., 2008; Nidzworski et al., 2014; Chang and Park, 2010; Hleli et al., 2006; Ali et al., 2021), assuring simplicity, sensitivity and specificity. In EIS, the formation of the antigen- antibody complex is monitored by analyzing the electrical resistance of the system. This method is suitable for the development of a biosensor, as it detects binding events on the transducer surface (Zhao et al., 2022; Lisdat and Schäfer, 2008; Zhao et al., 2022). More particularly, an electrochemical biosensor is a device consisting of a working electrode (WE), a reference electrode (RE) and a counter electrode (CE). The working electrode's material varies (metal, metal oxides, glassy carbon,carbon nanomaterials etc.) and it undergoes chemical modification upon immobilization of the biomolecule. These chemically modified electrodes (CME) have been proven to be very efficient biosensors for immunodetection (Lisdat and Schäfer, 2008). A really well- established protocol for the modification of gold electrodes includes activation with EDC/NHS (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/ N-hydroxysuccinimide) chemistry. EDC is a high watersoluble cross-linker that catalyzes the formation of amide bonds between carboxyl and amide groups. NHS is used to stabilize the active intermediates in coupling reactions, by formating carboxylate active ester groups (Keleştemur et al., 2017; Bartczak and Kanaras, 2011).

In this work, we describe the development of a highly accurate and selective electrochemical immunosensor for the rapid diagnosis of chronic Q fever. It is based on the immobilization of CBU_1718 (GroEL) (UniProtKB - P19421 (CH60_COXBU) protein onto a gold electrode modified by EDC/NHS. The direct interaction between the immobilized antigen and chronic Q fever antibodies is monitored by EIS. The high selectivity of the resulting biosensor over the chronic Q fever antibodies makes it an ideal system for the diagnosis of the infection.

Materials and methods

Antigen production

Production of the GroEL protein (CBU_1718) was achieved as recently described by Psaroulaki et al. (Psaroulaki et al., 2020). Briefly, the CBU_1718 gene was amplified from *C. burnetii* Nine Mile Phase I genomic DNA by PCR. The PCR product was then ligated into pET-22b(+) expression vector, and this vector was then transformed into *E. coli* BL21 (DE3) cells. For antigen expression, the transformed *E. coli* cells were cultured at 37 °C, and at an OD_{600} of 0.5 the protein expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were harvested four hours after induction. The isolation and purification of the protein were carried out using Ni-NTA affinity chromatography, as the protein carried the 6xHis tag at its C-terminus (Psaroulaki et al., 2020). The purity of the isolated protein was tested by 12 % acrylamide gel followed by Coomassie blue staining and Western blot using a monoclonal anti-poly-histidine alkaline phosphatase conjugated antibody (Sigma). Protein concentration was estimated by the BCA assay and the isolated protein was stored at −40 °C.

Antigen evaluation

The diagnostic ability of the isolated GroEL protein was tested by western blot and ELISA. Sera from patients positive with acute or chronic Q fever, as well as sera from healthy individuals were used to evaluate GroEL's effectiveness in chronic Q fever diagnosis. Sera were tested for IFA using commercially available IFA slides (Focus Diagnostics) that can test for both phase I and phase II IgM and IgG *C. burnetii* antibodies. The IFA testing was performed at the laboratory of Clinical Microbiology and Microbial Pathogenesis, National Reference Center for the laboratory diagnosis of *C. burnetii* infection. Sera of Patients suspected to have *C. burnetii* infection are received daily, while sera from healthy blood donors had been collected in past studies (Mathioudaki et al., 2021; Psaroulaki et al., 2020; Vranakis et al., 2019). Approval for the collection of samples from healthy individuals was obtained from the Ethics Committee of the University Hospital of Heraklion, Crete, Greece (10925/2018). All sera used in the particular study were obtained after 2018. Titers of ≥ 1/1,920 for IgG or 1/480 for IgG together with 1/200 for IgM were considered as cut-offs for acute phase infection; the cut-off of ≥ 1/1,024 was considered as positive for chronic Q fever. The cut-offs of acute infection were based on past studies of the laboratory (Psaroulaki et al., 2020), while that of chronic Q fever was based on the guidelines for the diagnosis of this infection. In the western blot assay, AP-conjugated anti-Human IgG was used as a secondary antibody, while the use of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT) developed a reaction with a colorful product.

ELISA was performed on microtiter flat bottom plates (96 wells, Corning Costar). The purified antigen was coated at the concentration of 1 μg/mL by incubating at 4 °C overnight in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH = 9.6). Plates were washed with PBST washing buffer (phosphate-buffered saline + 0.05 % v/v Tween 20), and the wells were blocked with blocking buffer (PBST with 1 % w/v bovine serum albumin) at 37 °C for 1 h. After the blocking step, the plates were incubated with sera from patients and from healthy individuals (1:500 in PBST) at 37 °C for 1 h. They were then washed three times with washing buffer and were incubated with rabbit anti-human IgG (Boster Biological Technology) with peroxidase as a conjugate (1:3000 in blocking buffer) at 37 °C for 1 h. Next, the wells were washed three times and were incubated with a solution of substrate/chromogen for 10 min (TMB Core, BioRad, California, USA). The colorimetric reaction was stopped by adding 0.5 M H_2SO_4, and the optical density was read at 490 nm on a microplate reader (Multiskan FC, Thermo Scientific, Ratastie Finland).

Gold electrode modification and antigen immobilization

Goldscreen- printed electrodes (of 4 mm diameter) (Methrom, disposable SPEs DRP-250AT) were cleaned electrochemically by Linear Sweep Voltammetry in PBS buffer (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, pH = 7.0). The voltage was chan-

ged between −2 to 2 V at the scan rate of 0.1 V/s, against a Ag/AgCl reference electrode while a Pt electrode was used as counter. The treated gold electrodes were then immersed overnight in 1 mM 11-mercaptoundecanoic acid (11-MUA) in ethanol to form a self-assembled monolayer (SAM) on the gold surface (Lisdat and Schäfer, 2008; Bürgi, 2015). Long alkane thiols can form well- ordered, closed packed monolayers on Au, due to the formation of the sulfur- gold bond (Bürgi, 2015). After this step, the electrodes were rinsed with ethanol to remove the unbounded thiols. Following this step, the activation of the terminal carboxylic groups occurred. For this purpose, the modified electrodes were treated with 1:1 v/v mixture of 0.4 M EDC-0.1 M NHS (MES buffer, pH = 5.0) for 2.5 h.

The recombinant protein was coupled following rinsing the gold electrodes with PBS and drying them in air. Antigen immobilization onto the gold electrode was optimised by altering several parameters such as protein concentration and incubation time. The resulting biosensor demonstrated its optimal performance when 4 μL of 10 μg/mL solution of purified GroEL was dropped- coated onto the surface of the gold electrode and the electrode was left overnight at 4 °C. The nonbinding antigen was then removed by rinsing with PBS (pH = 7.0) and water, and the electrodes were dried in air. Fig. 1 depicts the whole biosensor construction procedure.

EIS measurements

Electrochemical Impedance Spectroscopy (EIS) measurements were conducted to characterize both the modification steps of the gold electrode and the immobilization of the GroEL protein, as well as the response of the resulting biosensor to the Q fever antibodies. A three-electrode system was used: the modified gold electrode as the working electrode, a platinum counter electrode, and an Ag/AgCl reference electrode. All EIS measurements occurred using a PalmSens4 potentiostat equipped with a frequency response analyzer, in the frequency range between 0.2 Hz and 100 kHz using a sinusoidal excitation signal to a potential amplitude of 10 mV. The impedance data were recorded at a potential value of 0.0 mV. All measurements were performed in PBS buffer (pH = 7.0) at room temperature (25 ±1 °C). The impedance spectra were fitted using the PSTrace 5.8 software.

For the antigen–antibody interaction measurements, sera from healthy blood donors, from patients positive to chronic Q fever, and from patients positive to acute Q fever were used. The response of the three modified electrodes was tested against one of the three different sera (from a healthy blood donor, a patient positive to chronic Q fever, and a patient positive to acute Q fever. In more detail, patient serum was diluted (1:500) in PBS buffer (pH = 7.0) and EIS measurement was performed after 2 min of stirring, followed by 6 min at ease, 1 min of stirring and 1 min at ease. Fig. 2 illustrates a schematic representation of chronic Q fever diagnosis using the resulting impedimetric biosensor.

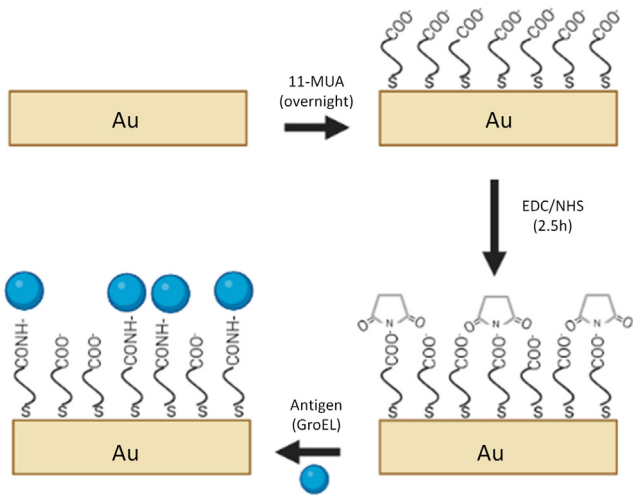

Fig. 1. GroEL protein immobilization via EDC/ NHS mediated coupling.

Results and discussion

Antigen preparation

Since GroEL (CBU_1718) showed higher yield in expression (0,65 mg/mL) compared to Com1, it was chosen for further examination.

The antigenic protein was tested for its quality, its quantity and its diagnostic ability before the development of the immunosensor. Fig. 3 validates that even after storage at −40 °C for two months, GroEL remains capable of differentially diagnosing Q fever disease in human serum. The results of western blot indicated that the isolated GroEL(Fig. 3-A to 3-D) reacted only with sera from patients positive to chronic Q fever (Fig. 3- E), while no reaction was observed with sera from healthy individuals or patients who suffer from the acute form of the disease (Fig. 3-F, 3-G). Additionally, as reported by Psaroulaki et al (Psaroulaki et al., 2020), sera from patients with chronic inflammatory rheumatic diseases (Systemic Lupus Erythromatosus, Rheumatoid Arthritis) were used as control to test potential cross reactions. ELISA measurements and statistical analysis confirmed that GroEL displays high sensitivity (96.4 %) and specificity (84.8 %) against chronic Q fever while its cut-off was set at 1.59 (Psaroulaki et al., 2020).

Biosensor development

Impedance spectroscopy is one of the most widely used methods for monitoring interactions with surfaces and self-assembled monolayers (Hou et al., 2006; Chen et al., 2006; Hnaien et al., 2008; Nidzworski et al., 2014; Chang and Park, 2010; Hleli et al., 2006; Ali et al., 2021; Manickam et al., 2010; Yun et al., 2007; Tlili et al., 2006; Choi et al., 2020). Since impedance is the measure of the total ability of a circuit to resist current, it considers all parameters inhibiting the flow within a resistor such as inductance, resistance, and capacitance. During EIS measurements, the electrical current, measured as an AC voltage, is applied to the sample at different frequencies. Impedance (Z) can then be calculated as the ratio of the frequency-dependent potential (E) to the frequency-dependent current (I).

As the impedance is expressed as a complex number ($Z_{(\omega)} = Z'_{(\omega)}$-$jZ''_{(\omega)}$), the data are often presented in a Nyquist plot, where the real part (Z') is on the x-axis, and the imaginary part (Z") on the y-axis. Usually this plot is demonstrated as a semicircle, which signifies a charge transfer process, while the size of the semicircle represents the amount of charge transfer resistance we have. However, Nyquist plot can also look like a straight line with positive slope, in the case of a diffusion process (Warburg impedance) (Chang and Park, 2010).

Fig. 4 shows the impedance Nyquist spectra of a gold electrode before and after it has been modified with GroEL protein. The diagram of the bare gold outlines a semi-circle, distinctive of a resistance connected in parallel with a capacitor and a linear part, which is present at low frequencies and which is due to the diffusion process (Warburg Impedance) (Tlili et al., 2006). However, after antigen immobilization onto the surface of the electrode, a pronounced high frequency charge transfer semicircle appears while the linear part is no longer observed. This suggests that the Warburg Impedance is absent as the protein GroEL electrically insulates the modified gold electrode surface (Chang and Park, 2010; Tlili et al., 2006).

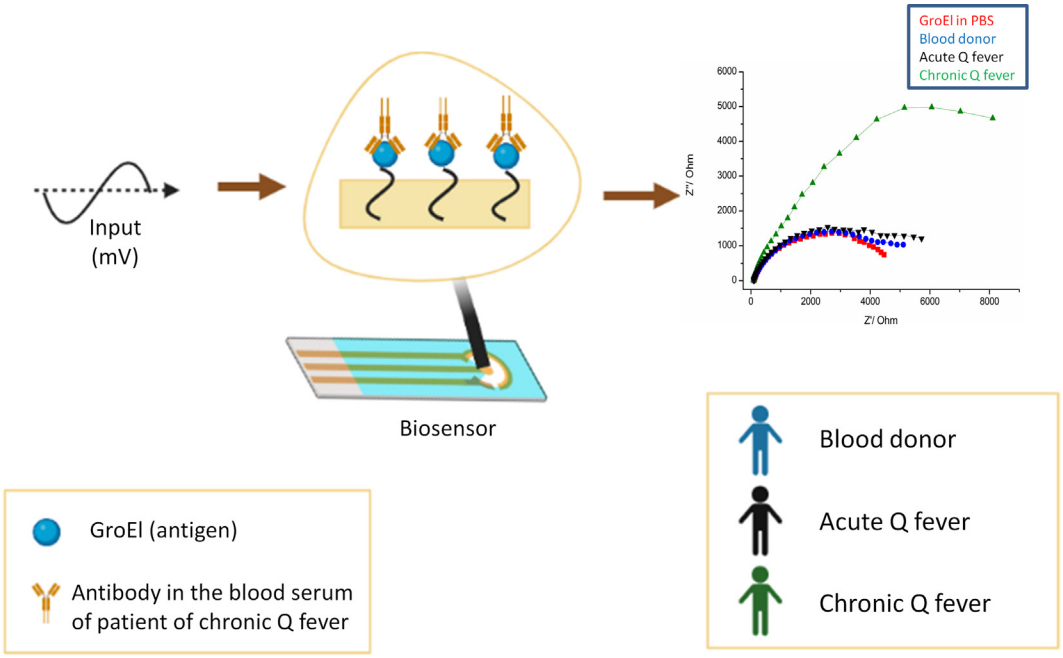

Fig. 2. Diagrammatic illustration of the EIS biosensor for Chronic Q fever detection.

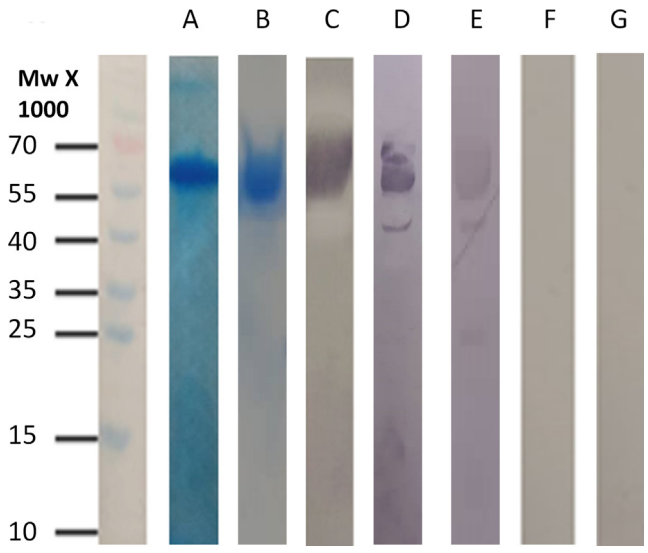

Fig. 3. **A.** SDS-PAGE for GroEL after affinity chromatography **B.** SDS-PAGE for GroEL after affinity chromatography and storage at −40 °C. **C.** Western blot using anti-poly-histidine alkaline phosphatase conjugated antibody. **D.** Western blot using anti-poly-histidine alkaline phosphatase conjugated antibody after storage at −40 °C. **E.** Western blot using serum from patient suffering from chronic Q fever. **F.** Western blot using serum from patient with acute Q fever. **G.** Western blot using serum from blood donor.

Biosensor evaluation

The protein GroEL was immobilized onto the modified gold electrode, and the response of the resulting biosensor to several serum samples was evaluated by EIS measurements. Fig. 5 shows the Nyquist plot (A) and the corresponding Bode (B) plot when sera from a healthy individual, one positive with acute Q fever and one with the chronic form of the disease are used. The EIS spectrum of the biosensor in PBS when no serum was added to the test solution is shown on the same graph for comparison. It was observed that when serum from a healthy individual and from a patient with acute Q fever were used, the EIS spectra showed no significant difference as the two plots overlap. On the other hand, when serum from a patient suffering from the chronic form of Q fever was used, the impedance spectrum changed dramatically. The data were fitted to the simplified Randles circuit (Fig. 6), where R_{ct} is the charge transfer resistance to be calculated by the diameter of the semicircle (Choi et al., 2020), R_1 is the electrolyte resistance and C_{dl} is the double layer capacitance. The calculated values for the above measurements are shown in Table 1.

It is usually the case that upon electrode functionalization with a biomolecule, the impedance increases (Cancelliere et al., 2021), as a result of an increased coverage of the electrode surface with a nonconductive organic material. Non-conductive material induces a slower electron transfer rate and higher charge transfer resistance as compared to the bare metal electrode (Cancelliere et al., 2021).

The data from Table 1 indicate that the values of both R_{ct} and C_{dl} increase at least two and fourfold, respectively, when the biosensor is exposed to chronic Q fever antibodies compared to that obtained in PBS buffer or acute Q fever patients. The increase in the Rct corresponds to the increase in the surface resistance on the sensor, while the increase in C_{dl} value can be attributed to the insulating effect of the antibody binding on the sensing surface. The large total impedance increase is consistent with the expansion of the non-conductive electrode surface coating, which is a result of the biorecognition of the chronic Q fever antibody with the immobilized selective protein. On the other hand, the calculated R_{ct} and C_{dl} values remained constant (Table 1) when sera from blood donors and acute Q fever patients were added.

When comparing EIS measurements results to those of ELISA (Psaroulaki et al., 2020), it must be pointed out that they follow a similar pattern. According to ELISA studies, GroEl demonstrates high sensitivity and specificity in the differential diagnosis of chronic Q fever. Following those findings and after the immobilization of GroEl onto gold surface, EIS measurements suggested that the antigen can selectively recognize chronic Q fever antibodies. This outcome is directly in line with ELISA results, suggesting the efficiency of the proposed biosensor.

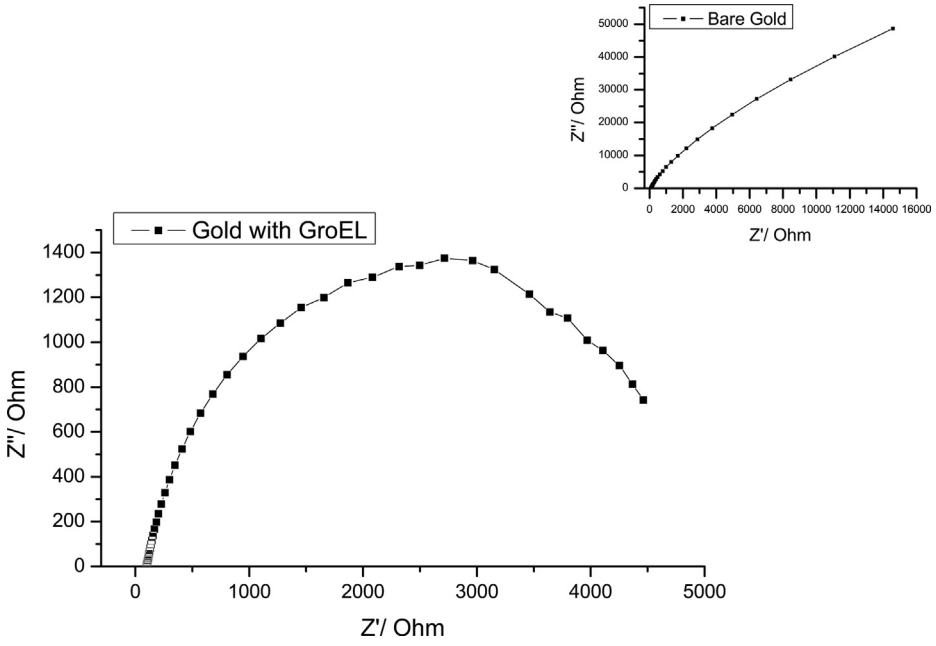

Fig. 4. Impedance Nyquist spectra of bare gold electrode (inset) and gold electrode modified with GroEL protein in PBS buffer. Potential amplitude is 10 mV and frequency range is 100 kHz–0.2 Hz. The impedance data were recorded at the potential of 0.0 mV.

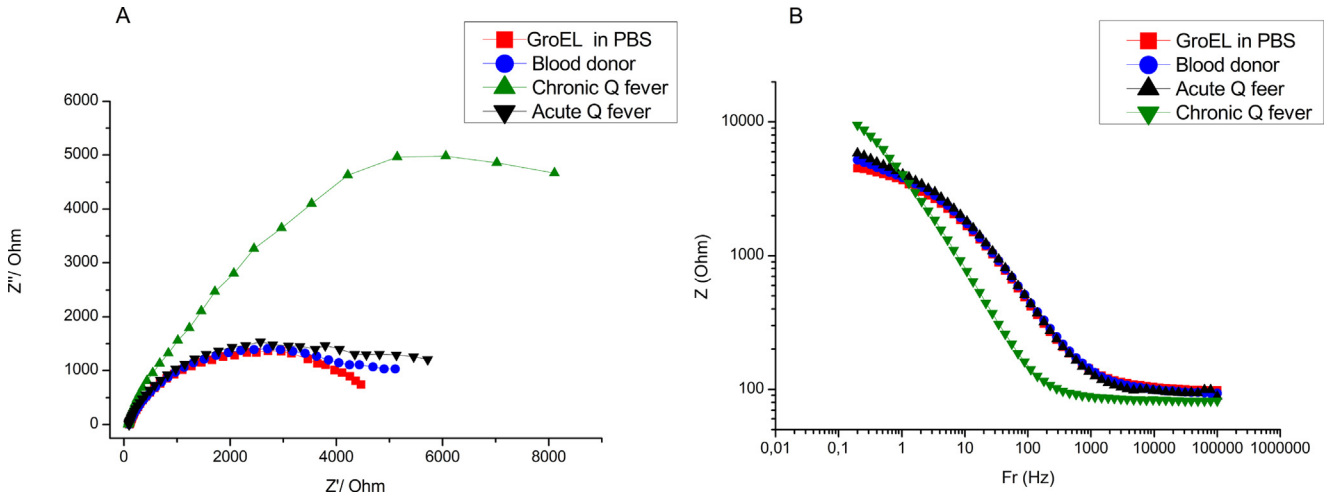

Fig. 5. A. Impedance Nyquist plot of GroEL modified electrode in PBS and in different patients' sera. Potential amplitude is 100 mV and frequency range is 100 kHz–0.2 Hz. The impedance data were recorded at the potential of 0.0 mV. **B.** Equivalent Bode plot of EIS biosensor against PBS and different patients' sera.

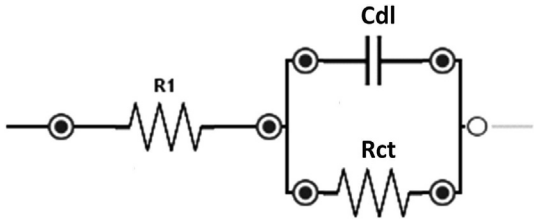

Fig. 6. The Randlers circuit is fitted by the data from EIS measurements.

The sensor-to-sensor reproducibility was evaluated, as shown in Fig. 5B. These data show the relationship between the Z values versus the frequency range used. It is evident that the large increase in impedance upon the antigen–antibody interaction is achieved at frequencies lower than 1.0 Hz. Fig. 7 depicts the mean total impedance for different sensors at 0.51 Hz, in PBS and different serum samples. All biosensors exhibit a very similar (RSD 2–3 %) total impedance increase when exposed to chronic Q fever antibodies, while no significant response was observed against healthy blood donors and acute Q fever sera. This result indicates that the developed GroEL biosensor could serve as a diagnostic tool with high specificity for chronic Q fever antibodies, allowing for an easy-to-use, direct and accurate determination of chronic Q fever ill patients.

Conclusions

In this work, we showed the development of an impedimetric biosensor based on highly selective *C. burnetii* antibodies for the direct diagnosis of chronic Q fever. The biosensor was developed via immobilization of the antigen onto a gold electrode using EDC/NHS immobilization methodology. The optimum detection was achieved using

Table 1
Calculated values for solution resistance R_1, polarization resistance R_{ct} and double layer capacitance C_{dl} for the impedance spectra are shown in Fig. 5.

	GroEL modified electrode in PBS	GroEL modified electrode in serum from acute Q fever patient	GroEL modified electrode in serum from blood donor	GroEL modified electrode in serum from chronic Q fever patient
R_1 (kΩ)	0.27	0.29	0.28	0.26
R_{ct} (kΩ)	3.7	4.4	4.0	10.3
C_{dl} (μF)	11.4	11.0	10.8	40.0

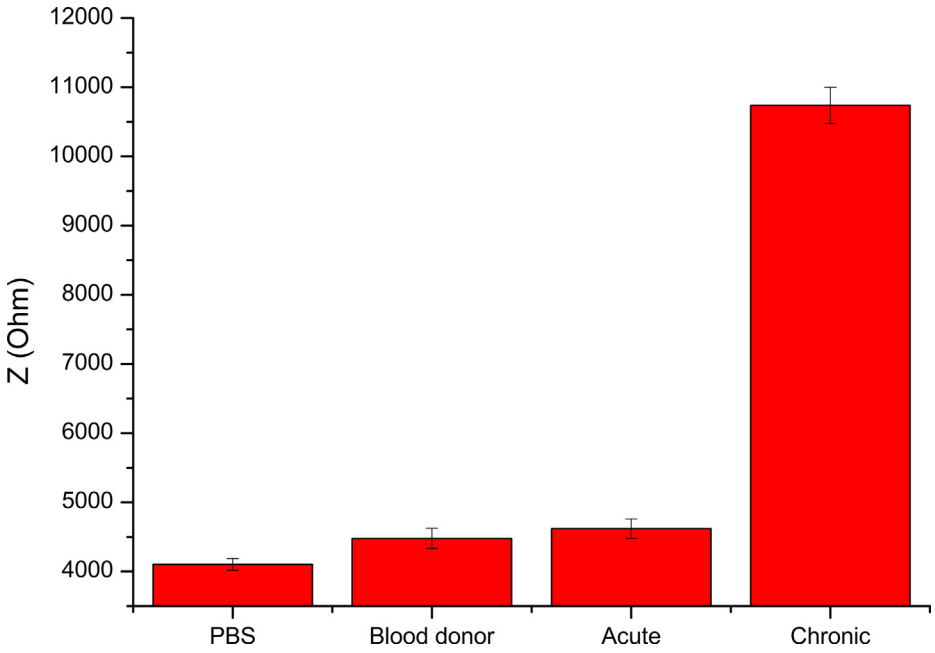

Fig. 7. Evaluation of sensing performance against the chronic form of Q fever. Total impedance (Z) was measured by EIS, which was performed in PBS buffer using different patients' sera. Potential amplitude was 100 mV and frequency range was 0,51 Hz. Evaluation was performed in triplicate (N = 3).

impedance measurements at frequencies below 1 Hz, at which point the biosensor showed its maximum sensitivity. The findings of the current research lay the basis for the development of a novel, easy- to –use and reliable immunosensor for the diagnosis of the worldwide zoonosis Q fever. In addition, the suggested biosensor showed the required discrimination ability allowing for the accurate serological detection of chronic Q fever, while the developed system can also be modified for the detection of other biomarkers, such as the ones from acute Q fever.

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CRediT authorship contribution statement

Eirini Mathioudaki: Conceptualization, Methodology, Software, Validation, Investigation, Data curation, Writing – original draft, Visualization. **Yiannis Alifragis:** Software, Data curation, Writing – review & editing. **Maria Fouskaki:** Validation, Formal analysis, Data curation, Writing – review & editing. **Dimosthenis Chochlakis:** Methodology, Writing – review & editing. **Hao Xie:** Methodology, Investigation, Writing – review & editing. **Anna Psaroulaki:** Resources, Writing – review & editing. **Georgios Tsiotis:** Conceptualization, Resources, Writing – review & editing, Visualization. **Nikolaos Chaniotakis:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Project administration.

Data availability

Data will be made available on request.

Declaration of Competing Interest

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

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Editorial Declaration

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