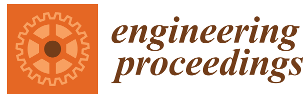

Proceeding Paper

A Capacitive Biosensor for the Early Detection of Pancreatic Cancer Using Carbohydrate Antigen 19-9 †

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Abstract: Pancreatic cancer has one of the highest cancer mortality rates, as it is often detected in late stages, when unresectable tumours are present. Researchers have identified a biomarker associated with the early detection of pancreatic cancer, called Carbohydrate Antigen 19-9 (CA19-9), and have recommended it for pancreatic cancer screening, and for the monitoring of the efficacy of pancreatic cancer treatments. The development of a biosensor for the detection of CA19-9 is discussed in this paper. The biosensor uses capacitive spectroscopy on gold interdigitated electrodes. This electrochemical transducer mechanism was selected as appropriate due to its increased popularity in point-of-care applications. Mouse monoclonal anti-CA19-9 antibodies were covalently bound to the gold surface using cysteamine hydrochloride and glutaraldehyde, and immobilization was verified with a Zeiss AxioObserver fluorescence microscope. Next, the antigen was prepared in different concentrations, and added to the prepared electrodes. Impedance spectroscopy was done using the PalmSens4 Electrochemical Interface, where five different concentrations of CA19-9 were detected in this process. The concentrations ranged from 10 U/ml to 300 U/ml, which includes the threshold concentration of CA19-9 for the detection of pancreatic cancer, of 37 U/ml. This biosensor is therefore suited to detect the CA19-9 concentrations needed for pancreatic cancer screening.

Keywords: biosensors; electrochemical impedance spectroscopy; pancreatic cancer

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1. Introduction

Pancreatic cancer has one of the leading cancer mortality rates worldwide, with an estimated 5-year survival rate of about 5% [1]. The high incidence-to-mortality ratio can be attributed to pancreatic cancer being detected in late stages, when the tumors are unresectable. Carbohydrate Antigen 19-9 (CA19-9) has been identified as a biomarker associated with early detection of pancreatic cancer [2], where a concentration above 37 U/ml is considered useful for its diagnostic value for pancreatic cancer [3]. Current CA19-9 tests use an ELISA (enzyme-linked immunosorbent assay) or radioimmunoassay [4], which are resource intensive processes as they require laboratory analyses. As an alternative to these tests electrochemical biosensors have recently gained popularity as point-of-care (POC) devices, due to its potential to simplify the testing process for various biomarkers. These biosensors use various electrochemical transducer mechanisms which directly convert a biological event to an electrical signal, including voltammetry, amperometry and electrochemical impedance spectroscopy (EIS). EIS uses a sinusoidal perturbation to a two-electrode electrochemical system, and obtains measurements of complex impedance as a function of frequency [5]. This can mathematically be related to complex capacitance, which is represented by C' and C'', its real and imaginary components respectively. The real component represents the real double layer capacitance at steady state, and the imaginary component relates to dielectric loss [6]. An electrochemical biosensor that utilizes capacitance spectroscopy will

therefore be able to detect changes on the surface of an electrode as a result of biological events. A biosensor using complex capacitance can be useful in POC detection of CA19-9, simplifying its testing process. This study uses electrochemical impedance spectroscopy to derive complex capacitance, which is shown to vary with different concentrations of CA19-9. This biosensor has various components which make unique contributions to the field, including the interdigitated electrode fabrication using printed circuit board, and immobilization of CA19-9 monoclonal antibodies using cysteamine hydrochloride and glutaraldehyde. Most importantly, it describes the functionality of a novel proof-of-concept biosensor using complex capacitance, which differentiates between concentrations above and below the threshold for the early detection of pancreatic cancer.

2. Materials and Methods

Gold interdigitated electrodes (IDE) were used as a sensor substrate as part of the electrochemical transducer mechanism in this study. IDEs using printed circuit board (PCB) were designed, and manufactured with electroless nickel immersion gold (ENIG) to ensure a layer of gold to which antibodies could bind. These electrodes were fabricated on a standard FR4 PCB sheet, as shown in Figure 1, with a width and spacing of 130 μm in the interdigitated region.

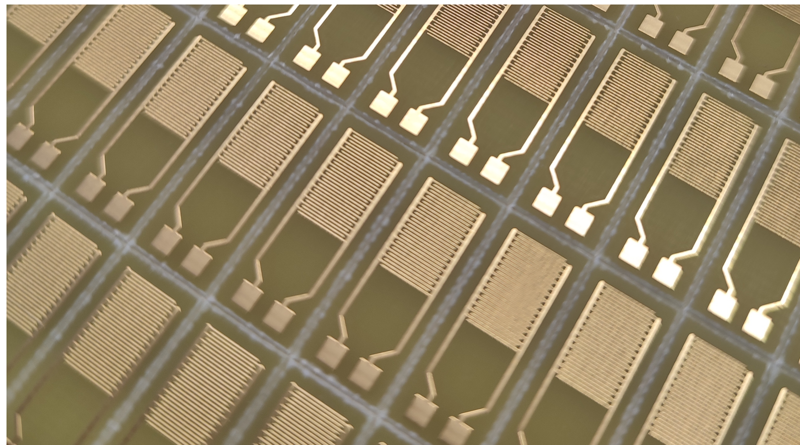


Figure 1. Sheet of interdigitated electrodes with a width and spacing of 130 μm.

These electrodes were rinsed with 70% ethanol and deionised water, and placed in an oven at 50 °C until dry. This was followed by ten minutes in a Jelight UVO cleaner (Jelight Co. Inc., CA, USA), to ensure that all biological material was removed from the gold surface of the electrodes. The UVO cleaner used ultraviolet light with ozone, and allowed for preparation of the gold surface for the next step, which was antibody immobilization.

A standard immobilization protocol to immobilize antibodies to the gold surface of the IDE was adapted for this project [7]. All reagents and proteins used for immobilization and antigen testing are shown in Table 1. First, cysteamine was used to functionalize the gold surface of the IDEs due to its thiol (-SH) and amine (-NH2) moieties, as well as gold's high affinity for thiol binding. Next, glutaraldehyde was used as a crosslinking agent as it's aldehyde group (-CHO) is used to bind to the free amines formed with the cysteamine and the amines on the antibodies. The third and final step is the addition of the antibodies. This immobilization procedure is shown graphically in Figure 2.

Table 1. Reagents and proteins used for all incubation steps.

	Supplier	Product/CAS Number	Pack Size
Cysteamine HCl	Sigma Aldrich	CAS: 156-57-0	25 g
Glutaraldehyde	Sigma Aldrich	CAS: 111-30-8	100 ml
Anti-CA19-9 Ab	ThermoFisher	MA1-19211	100 µl (1 mg/ml))
CA19-9 Protein	RayBiotech	227-20044	50KU (69,343 U/ml)
FITC Isomer 1	ThermoFisher	CAS: 3326-32-7	10 mg

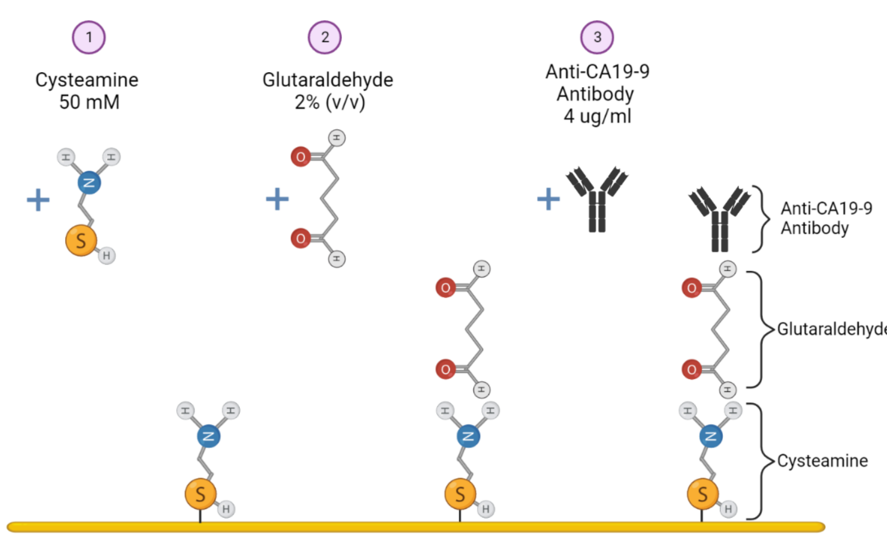


Figure 2. Immobilization using Cysteamine and Glutaraldehyde (Created in BioRender.com).

Antibody immobilization was validated by attaching a fluorescent marker to the antibody, and imaging the electrode using a fluorescence microscope. Since FR4 board auto-fluoresces, two DropSens gold IDEs on a glass substrate were prepared using the same antibody immobilization process, and used for fluorescence microscopy validation. Once the immobilization process had been completed and validated, antigen tests commenced.

2.1. Antibody Immobilization

15 IDEs with FR4 substrate were prepared for antibody immobilization, and a volume of 300 µl was used for each electrode for each incubation step. Electrodes were rinsed with deionised water in between steps, and samples and solutions were kept at 4 °C.

The immobilization protocol and antigen concentration testing procedure were as follows:

1. Cysteamine was prepared in 70% ethanol, at a concentration of 50 mM, and electrodes were incubated overnight, in the dark.
2. Glutaraldehyde was prepared in deionised water, at 2% (*v*/*v*), and incubated for one hour, in the dark.
3. Mouse monoclonal anti-CA19-9 antibodies were diluted in phosphate buffered saline (PBS) at pH 7.4 for 6 hours.
4. Electrodes were rinsed with PBS after antibody incubation, and impedance spectroscopy was run to obtain baseline measurements.

Two DropSens gold IDEs on glass substrates were also prepared using the protocol above, to be used for validation of the immobilization procedure and antibody binding to the gold. After the baseline tests, the ten most consistent electrodes were selected for antigen tests.

2.2. Validation of Antibody Immobilization

Antibody immobilization was validated by addition of a fluorescent tag to the prepared electrodes. Fluorescein Isothiocyanate (FITC) is a common fluorophore used to tag antibodies for various fluorescence applications. FITC tagging was therefore used by adhering to a standard protocol, and the process was as follows:

1. FITC powder was diluted in a 5mg/ml concentration in 70% ethanol to form a stock solution.
2. The stock solution was diluted in a ratio of 1:50 in a sodium bicarbonate buffer at pH 9.0.
3. Electrodes were incubated in the FITC solution, in the dark at 4 °C, for three hours.
4. Electrodes were rinsed in TBST (tris-buffered saline with Tween 20) to remove any unbound FITC, and were kept in the dark, in TBST prior to imaging.

Fluorescence microscopy was used to validate the presence of antibody binding using the FITC and a Zeiss AxioObserver wide field fluorescence microscope. The microscope was coupled with a Colibri 7 multicolor LED light source, with the ability to deliver up to seven excitation wavelengths, including the 495 nm excitation wavelength of FITC. The excitation wavelength, exposure and light intensity were controlled using the Zen Blue software for the microscope, and images of the immobilized electrodes were compared to control samples, which had no antibody immobilized, to show that the immobilization procedure was successful.

2.3. Antigen Incubation and Testing

The antigen concentrations used for incubation were based on concentrations of interest and thresholds found in literature. A concentration of 37 U/ml or less is always considered below the threshold of concern for pancreatic cancer [3]. Other studies have found 150 U/ml to be a better cutoff concentration for identifying resectable tumors [8]. Antigen concentrations tested were therefore 10 U/ml, 37 U/ml, 100 U/ml, 150 U/ml and 300 U/ml. Antigen was prepared in five concentrations by diluting the stock solution in PBS, and incubated for 40 minutes. Two electrodes were prepared per concentration, for each of the five dilutions. Electrodes were rinsed with PBS after antigen incubation and impedance spectroscopy was run again to obtain final measurements.

All electrical measurements were recorded using the PalmSens4 Electrochemical Interface, and were run at ten frequencies. Ten frequencies were selected between 500 Hz and 1600 Hz, in accordance with frequency ranges of interest for biosensor applications [9], and specifically for CA19-9 [10]. Results were obtained in the form of impedance magnitude and phase and were processed to obtain real and imaginary components of impedance and capacitance respectively. The real and imaginary components of the complex capacitance are given by

$$C' = \frac{Z''}{\omega(Z'^2 + Z''^2)} \quad (1)$$

and

$$C'' = \frac{Z'}{\omega(Z'^2 + Z''^2)} \quad (2)$$

where Z' and Z'' are the real and imaginary components of the complex capacitance, and ω is the angular frequency [6]. The complex capacitance was computed for each frequency and analyzed for each sample.

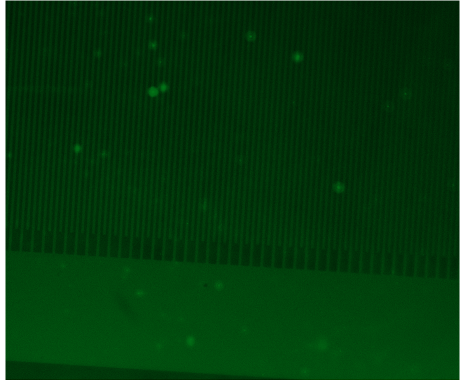
3. Results

The results obtained during this study include fluorescence microscopy images to show that immobilization was validated, as well as results of the impedance spectroscopy

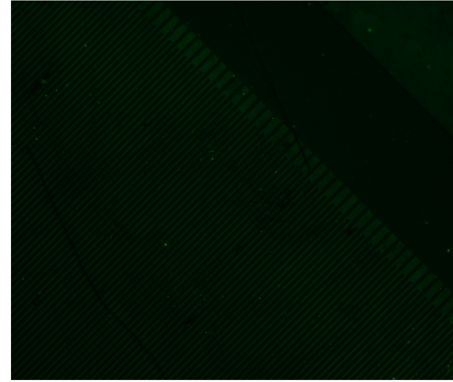
and derived capacitance spectroscopy, which show an increase in real capacitance as the antigen concentration increases.

3.1. Microscopy

Figure 3a shows an image of an IDE which underwent the Cysteamine and Glutaraldehyde immobilization process, and clearly shows fluorescence on the gold. The image was taken at the edge of the electrode to confirm that it was the gold regions that fluoresced, and not FITC that bound or adhered to the glass. Figure 3b shows a control sample, with little to no fluorescence, as expected.



(**a**) IDE with immobilized antibody



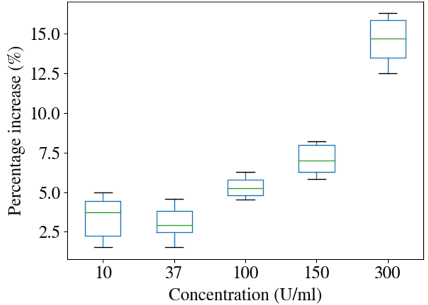
(**b**) Control IDE without immobilized antibody

Figure 3. Microscopy results validating antibody immobilization.

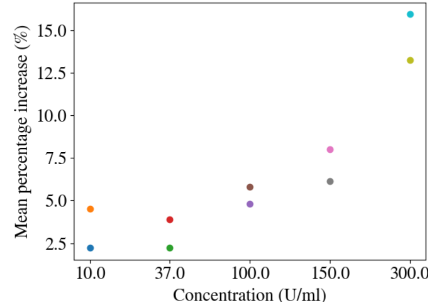
These images therefore provide validation that the immobilization process was successful, and that the remaining electrodes can be used for antigen tests.

3.2. Results from Antigen Tests

Impedance measurements after the antigen incubation period were compared to the measurements before, and the percentage change in the imaginary components of impedance and capacitance were related to the presence of various concentrations of the CA19-9 protein. Furthermore, the frequency range was narrowed to the optimal four frequencies, between 700 Hz and 1000 Hz. This allowed for increased accuracy in differentiation between concentrations by measuring the average real capacitance over these frequencies. This frequency range is small since a narrow bandwidth is needed to represent information about the change in the real double layer capacitance, as a result of binding events on the surface of the gold. Figure 4a shows a box and whisker representation of the real capacitance at all frequencies for all samples at their respective frequencies. Figure 4b show the average real capacitance per electrode, where each data point represents one electrode over all frequencies. This shows an increase in real capacitance with an increase in antigen concentration.



(**a**) Samples over all frequencies



(**b**) Average real capacitance for each electrode

Figure 4. Antigen tests results showing variation in real capacitance for different concentrations.

4. Discussion

The boxes between the 10 U/ml and 37 U/ml overlap, which is acceptable since concentrations below 37 U/ml are considered safe, and not relevant in the early detection of pancreatic cancer. The boxes of the remaining concentrations do not overlap with one another, showing distinction between concentrations. Similarly, in Figure 4, the average real capacitance for each of the samples at the different concentrations do not overlap, apart from the one sample at 10 U/ml. Thus average real capacitance is a good indication of the ability to distinguish between concentrations as it directly shows the parameter that would be used in a point-of-care device. This study therefore successfully demonstrated the potential for a capacitive biosensor to detect diagnostic concentrations of CA19-9, using EIS as a transducer mechanism.

Future work could include repetition of these tests on a bigger scale, a comparison between different IDEs for the detection of CA19-9, and implementation of machine learning to better optimize the frequency range and relevant parameters. These tests were also conducted using purified antigen samples, and could be expanded to use patient blood samples in future, for use in POC settings.

Author Contributions: T.E. contributed to conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing-original draft preparation, visualization and funding acquisition; W.P. contributed to conceptualization, writing-review and editing, resources, supervision, project administration and funding acquisition; A.-M.E. contributed to conceptualization, writing-review and editing, resources, supervision and project administration. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CA19-9	Carbohydrate antigen 19-9
POC	Point-of-care
EIS	Electrochemical impedance spectroscopy
IDE	Interdigitated electrode
FITC	Fluorescein isothiocyanate

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