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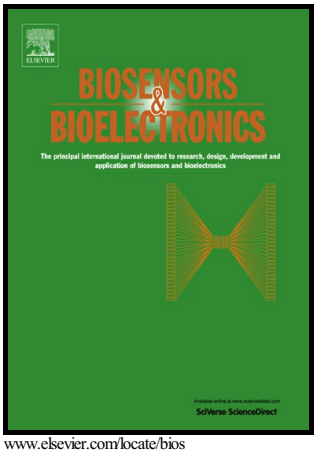

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Sensitive detection of *Campylobacter jejuni* using nanoparticles enhanced QCM sensor

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Abstract

A quartz crystal microbalance (QCM) sensor platform was used to develop an immunosensor for the detection of food pathogen *Campylobacter jejuni*. Rabbit polyclonal antibodies and commercially available mouse monoclonal antibodies against *C. jejuni* were investigated to construct direct, sandwich and gold-nanoparticles (AuNPs) amplified sandwich assays. The performance of the QCM immunosensor developed using sandwich assay by utilising the rabbit polyclonal antibody as the capture antibody and conjugated to AuNPs as the detection antibody gave the highest sensitivity. This sensor achieved a limit of detection (LOD) of 150 colony forming unit (CFU) mL^{-1} of *C. jejuni* in solution. The QCM sensor showed excellent sensitivity and specificity for *Campylobacter* detection with low cross reactivity for other foodborne pathogens such as Salmonella *Typhimurium*, (7%) *Listeria monocytogenes* (3%) and *Escherichia coli* (0%). The development of this biosensor would help in the sensitive detection of *Campylobacter* which can result in reducing pre-enrichment steps; hence, reducing assay time. This work demonstrates the potential of this technology for the development of a rapid and sensitive detection method for *C. jejuni*.

Keywords: *Campylobacter jejuni*, Biosensors, Quartz crystal microbalance (QCM), Foodborne illness, Nanoparticles

1. Introduction

Campylobacteriosis is a foodborne illness caused by bacteria of the genus *Campylobacter* (Vencia et al., 2014). A steady increase was recorded in 2012 for *Campylobacter* incidence

rate in European Union (EU) countries including Poland, Czech Republic, Slovakia, Hungary, Romania, Bulgaria, Greece, Portugal, Spain, France and Ireland showing a notification rate higher than 55.49 per 100,000 population (EFSA and ECDC, 2014). Approximately 214,268 cases of foodborne illness and around 100,000 deaths due to *Campylobacter* spp. infection in the European Union countries have been reported in 2012 (EFSA and ECDC, 2014). Since 2010, *Campylobacter* has displaced *Salmonella* as the most frequently reported factor or causative agent in foodborne related outbreaks in the UK representing 18 outbreak cases amounting to 30% of the 61 total cases (Public Health England, 2011). The EU Commission Regulation (EC) No 2073/2005 on the microbiological criteria aimed at foodstuffs in 2005 mandates that food products should not contain microbes, their metabolites or toxins in quantities that produce unacceptable health risk to human. In another part of the regulation specifically in Article 4 of EU Regulation (EC) No 852/2004, food business operators needs to comply with standards pertaining to microbiological criteria. The criteria include adhering to stipulated protocols and instructions given by the competent authority in activities that cover testing of samples, follow up analyses and execution of corrective actions if any (Roasto et al., 2012).

Screening for *Campylobacter* is routinely carried out globally with numerous quantification methods which are commercially available for detection of the pathogen in food products. They include culturing, microscopy, enumeration methods and biochemical testing such as polymerase chain reaction (PCR), immunoassays and deoxyribonucleic acid (DNA) probes (Yang et al., 2013). Several of these methods are sensitive and rapid but suffer from setbacks such are expensive, requiring extensive sample preparation, have poor selectivity and time-consuming. Since most poultry-based products are consumed within days of production, this presents a challenge for available methods as by the time the positive confirmation has been made the population would be exposed to *Campylobacter* leading to outbreaks of foodborne illness (Che et al., 2001). Due to these shortcomings, researchers around the world have been searching for fast and rapid methods of detection. The prime candidate that can fulfil these goals is biosensor technology. This technology provides cost effective, rapid, sensitive and reliable diagnostic approaches without time consuming procedures. One of the most sensitive immunosensor methods reported to date for microbial detection is QCM.

A wide range of limit of detection (LOD) have been reported for QCM immunosensors for the rapid and specific detection of different bacteria such as *Salmonella* Typhimurium at 10-20 CFU mL^{-1} (Salam et al., 2013), *Escherichia coli* O157:H7 at 10 CFU mL^{-1} (Guo et al., 2012), *Pseudomonas aeruginosa* at 1.5×10^2 CFU mL^{-1} (Cai et al., 2011) and *Staphylococcus epidermidis* at 1.3×10^3 CFU mL^{-1} (Xia et al., 2008). To the best of our knowledge, the use of QCM for the detection of *C. jejuni* was reported by Yakovleva et al. (2011) and by Safina et al. (2008) as a method to discriminate serostrains level. Both works utilise lectins as the capturing ligands. Yakovleva et al. (2011) characterised seven *C. jejuni* strains using four commercially available lectins as capturing ligand on a QCM. The system is able to discriminate *C. jejuni* strains proving that the QCM technique is a powerful tool. However, no cross reactivity works and the sensitivity of the detection were reported. In the works of Safina et al. (2011), several *C. jejuni* strains were tested using lectins and they reported a detection level of 10^3 CFU mL^{-1}.

In this study, a fully automated QCMA-1 device was employed for the first time for the detection of *C. jejuni* using both monoclonal and polyclonal antibody systems coupled with the use of gold nanoparticles (AuNPs) to increase the sensitivity. Different assay formats (direct and sandwich) for *C. jejuni* detection were investigated and each assay sensitivity was assessed through the construction of *C. jejuni* standards plots. Antibody-conjugated AuNPs was examined for its mass amplification effect to improve the sensitivity of the QCM immunosensor for the detection of *C. jejuni* in food samples. Finally, specificity of the assay was validated by cross reactivity studies using other foodborne pathogens.

2. Materials and Methods

2.1. Materials and Reagents

Rabbit polyclonal antibody against *C. jejuni* was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. The mouse monoclonal antibody against *C. jejuni* (7721) and mouse IgG were purchased from Abcam Ltd, UK. 11-mercaptoundecanoic acid (11-MUDA), bovine serum albumin (BSA), ethanolamine hydrocloride, sodium acetate, phosphate buffered saline tablet (PBS; 10 mM, pH 7.4), 40 nm

gold colloidal (AuNPs), sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2) and ethanol were purchased from Sigma Aldrich, UK. N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Thermo Scientific, UK.

2.2. Instrumentation

A fully automated QCMA-1 biosensor system (Sierra Sensors GmbH, Germany) was employed in this work with its gold coated QCMA-1 sensor chips (20 MHz). The system enables the simultaneous measurement of active and control sensor surfaces with its two separate spots. The operating temperature and the flow rate of the running buffer during the assays were 25 °C and 25 µL min^{-1}, respectively. The data presented in this work are the averages of 3 data points for the assays described unless otherwise stated.

2.3. Bacterial strains

Campylobacter jejuni subsp. *jejuni* ATCC® 33291 (Oxoid Ltd, UK) was purchased from Fischer Scientific, UK. *Listeria monocytogenes, Salmonella* Typhimurium and *E. coli* O157:H7 were obtained from the culture collection of the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. These bacteria were used to verify the selectivity of the developed sensor. MacConkey sorbitol agar, Oxford medium and Xylose lysine deoxycholate agar (XLD) were purchased from Acumedia Manufacturers Inc., Baltimore, MD, and used for the enumeration of *E. coli* O157:H7, *L. monocytogenes* and *S.* Typhimurium, respectively.

2.4. Preparation of *C. jejuni* cells

Campylobacter jejuni subsp. *jejuni* ATCC® 33291 was used as a standard reference for *C. jejuni* detection. The *C. jejuni* inoculum was grown under microaerophilic conditions (5% O_2, 10% CO_2 and 85% N_2) on Campy cefex agar (Acumedia Manufacturers Inc., Baltimore, MD) in an anaerojar (Oxoid Ltd, UK) at 42 °C for 48 h. The preparation of the *C. jejuni* inoculum was carried out by sub-culturing a 48 h culture plate into Bolton broth (10 mL) in a 25 mL universal bottle. The inoculum was then grown at 42 °C for 48 h under microaerophilic conditions. After the 48 h incubation period, approximately 10 mL of the Bolton broth was then transferred into 90 mL of Bolton broth in a Duran bottle and further incubated at 42 °C for another 48 h under microaerophilic conditions. Cells were harvested through centrifugation at 10,000 x g for 15 min at 4 °C on a benchtop centrifuge (Sorvall™ Legend™ X1, Thermo Scientific, US). The pelleted cells were then washed with PBS and the

procedures were repeated three times. The bacterial cells were then resuspended in PBS and the bacterial suspensions were adjusted to optical densities (OD) at 600 nm of between 1.6 and 2.0 to obtain bacterial concentrations between 10^8 and 10^{10} CFU mL^{-1} on a UV/VIS spectrophotometer (Perkin-Elmer Lambda 20 GenTech Scientific, Inc. USA). The bacterial concentrations were confirmed by spread plate method. Colony forming units (CFU) on the agar plates were then counted as CFU mL^{-1}. The entire culture was then heat-killed at 70 $^{\circ}$C for 30 min. To ensure that the cells were dead, the cells were inoculated again on Campy Cefex agar and incubated at 42 $^{\circ}$C for 48 h (under microaerophilic conditions). Bacterial suspensions were then diluted to the desired target concentrations ranging between 10^1 and 10^9 CFU mL^{-1} in PBS. The numbers of cells in each dilution were then used for plotting the *C. jejuni* standard curve of the QCM immunosensor.

2.5. Sensor chip cleaning and self-assembled monolayer (SAM) deposition

The cleaning of the gold sensor chip was carried out with a piranha solution. The chip was immersed for 20 min in a piranha solution (H_2SO_4:H_2O_2/3:1) and then rinsed thoroughly with distilled water (Kallempudi et al., 2012). The sensor chip was then immediately immersed in 20 mM solution of thiol (11-MUDA) which was initially prepared in absolute ethanol. The chip was allowed to stand for overnight at ambient temperature in the solution to ensure an optimal formation of carboxy-terminated thiol layer. Immediately prior to use, the sensor chip was rinsed with ethanol and with deionised water and finally dried with nitrogen gas.

2.6. Surface activation and antibody immobilisation

The sensor chip was docked in the QCMA-1 chamber and then equilibrated with 10 mM of PBS (pH 7.4) at a constant flow rate of 25 μL min^{-1}. A filtered and degassed PBS buffer was used as a running buffer throughout the experiments. After a stable baseline had been achieved, the sensor chip was ready to be used for immobilisation events. Activation of the sensor surface was carried out utilising a 1:1 mixture of 400 mM EDC and 100 mM NHS prepared in deionised water filtered through a 0.22 μm filter membrane (Altintas et al., 2012). The mixture was mixed immediately prior to use and injected across the sensor surfaces to convert the carboxylic terminal groups into an active ester of NHS.

The gold sensor surface was activated with EDC-NHS solution for a total duration of 3 min at the flow rate of 25 μL min^{-1}. This was followed by the injection for 3 min (75 μl) of

different concentrations of rabbit polyclonal and mouse monoclonal antibodies against *C. jejuni* on the two parallel spots ranging from 10 to 100 µg mL^{-1} and 50 to 70 µg mL^{-1}, respectively. The antibodies were prepared in sodium acetate buffer (100 mM, pH 5.0). After the determination of the optimal capture antibody concentration for *C.jejuni* assays, a 70 µg mL^{-1} concentration of the capture and control (mouse IgG) antibodies were injected onto the two separate spots of the sensor for 3 min (75 µl). Blocking of both sensor spots were then carried out with 50 µg mL^{-1} of BSA in PBS (75 µl) for 3 min. Finally, capping of unreacted NHS esters was carried out by utilising 1 M ethanolamine, pH 8.5 for 3 min (75 µl).

2.7. Characterisation of SAM coated sensor chips by AFM

Sensor chips at various stages of the assay including bare gold, antibody immobilisation (70 µg mL^{-1}) and *C. jejuni* binding (10^5 CFU mL^{-1}) were visualised using atomic force microscope (AFM) (Dimension 3100 Scanning Probe Microscope, Bruker Ltd, UK). AFM analysis for the antibody immobilisation on the SAM-coated sensor surface and *C. jejuni* cells that were captured by the rabbit polyclonal antibody were carried out after having completed several cycles of buffer wash flow. The chips were undocked from the QCMA-1 instrument, dried in the air and then immediately placed in a glass petri dish. Each AFM image was scanned in tapping mode.

2.8. Optimisation of *C. jejuni* detection

Three different assays formats were used in this work including direct, sandwich and sandwich assay with antibody functionalised AuNPs. The direct detection assay was conducted by the injection of different concentrations of *C. jejuni* cells suspension prepared in PBS (10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 CFU mL^{-1}) over *C. jejuni* capture polyclonal or monoclonal antibodies (active channel), and mouse IgG (control channel) functionalised sensor surfaces. After the binding of *C. jejuni*, the surface was regenerated by the injection of a regeneration solution consisting of 100 mM HCl (1 minute, 25 µL) to regenerate the surface, or before a sandwich assay was performed. The pH of the regeneration solution was selected as 2.5 after optimisation studies and it was observed that the mildest regeneration conditions with short contact time did not lead to lose biological active function of the ligand (Supplementary document, **Fig.S1**)

The sandwich detection assay was carried out after the injection of *C. jejuni* cells to the surface of the sensor via the *C. jejuni* capture polyclonal antibody (active channel) and

over mouse IgG functionalised sensor surfaces (control channel). This was followed by injecting 75 μL of either *C. jejuni* monoclonal or polyclonal antibody (100 μg mL^{-1}) as detection antibodies for 3 minutes to allow binding. Calibration curve was prepared by injecting different concentrations of *C. jejuni* cell suspensions (10^4, 10^5, 10^6, 10^7, and 10^8 CFU mL^{-1}) for 3 minutes to allow binding interactions (75 μL).

2.9. Modification of AuNPs with anti-*C. jejuni* detection antibody

The preparation of the antibody-colloidal gold conjugate was according to the method described by Uludağ and Tothill (2012) and Zhao-Peng et al. (2007). The antibody-conjugated AuNPs was prepared by adding 100 μL of rabbit polyclonal antibody or mouse monoclonal antibody (50 μg mL^{-1}) to 1 mL of pH adjusted AuNPs solution (the pH was adjusted with 0.2 M NaOH solution to pH 9.0) followed by slow shaking at room temperature for 1 hour. A 100 μL of 10% BSA solution was then added to the antibody-AuNPs mixture and incubated for another 1 hour at room temperature. The conjugate was then centrifuged at 10,000 x g for 10 min. After centrifugation, two phases were obtained where the top layer was a clear supernatant and a dark red pellet which was loosely packed sediment of the antibody-conjugate AuNPs. The supernatant solution was discarded and the soft sediment of antibody-conjugate AuNPs was resuspended in 70 μL of PBS and stored at 4 $^{\circ}$C as a conjugate stock. The measurement of antibody-AuNPs conjugation was carried out through dynamic light scattering (DLS) technique (Malvern Zetasizer, Malvern Instruments Ltd, UK). The measurements were carried out in disposable cuvettes with a sample volume of 1100 μL according to the manufacturer's instruction. Each sample was measured in triplicate and the mean value was reported.

2.10. Sandwich detection assay with antibody modified AuNPs

A 20x dilution of the antibody-conjugated AuNPs stock in PBS was used in the entire *C. jejuni* binding assay. The sandwich detection assay was carried out after the injection of *C. jejuni* cells to the surface of the sensor via the *C. jejuni* capture of either polyclonal or monoclonal antibody (active channel). This was followed by a 75 μL injection of antibody-conjugated AuNPs to allow binding. Finally, an injection of a regeneration solution of 100 mM HCl (1 minute, 25 μL) was carried out to regenerate the surface and remove the bacteria from the immobilised capture antibody on the sensor chip. Calibration curve was prepared by

injecting different concentrations of *C. jejuni* cell suspensions (10^1, 10^2, 10^3, 10^4, and 10^5 CFU mL^{-1}) for 3 minutes (75 µL) to allow binding interactions to occur.

2.11. Specificity of the assay to *C. jejuni*

The specificity of the developed immunosensor assay was tested using Gram negative bacteria (*Salmonella* Typhimurium and *E. coli* O157:H7) and a Gram positive bacterium (*Listeria monocytogenes*). PBS was included as the negative control of this experiment. In the specificity assay, the sandwich assay with AuNPs was employed. Briefly, a 75 µl of cell suspension (1.0×10^5 CFU mL^{-1}) containing each different bacteria was injected on the sensor surface. Regeneration after injection of each bacteria suspension was carried out using 25 µl of 100 mM HCl.

2.12. Limits of detection

Calibration curves were fitted with a non-linear regression using four parameter logistic equations (Tijssen, 1985, Karpinski, 1990) as follow;

$$y = \frac{a-d}{1+\left(\frac{x}{c}\right)^b} + d$$

Where y is the response signal obtained (Hz), a and d the maximum and minimum signal response (Hz) of calibration curve respectively, x is the concentration of bacterial cells (Log CFU mL^{-1}) that produced a 50% signal response (EC_{50}) value and c bacterial cell concentration (Log CFU mL^{-1}) and b is the slope-like parameter (Hill coefficient), respectively, The limit of detection (LOD) was calculated as the average value of absorbance at blank concentration of bacteria at 3 standard deviations (SD). LOD and regression analysis were calculated using four-parameter logistics model available from PRISM non-linear regression analysis software from www.graphpad.com.

3. Results and Discussions

3.1. Immobilisation of the antibody on SAM

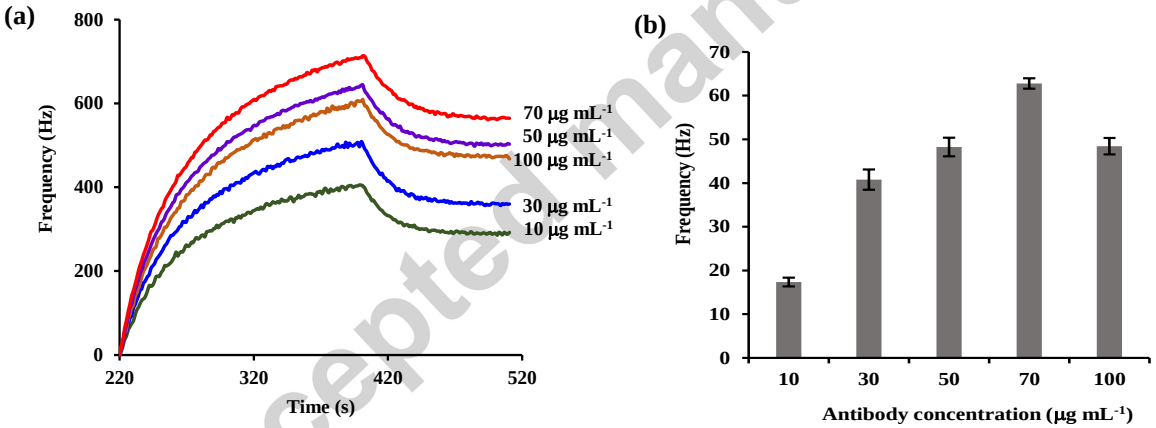
The optimal concentration of antibody immobilised on the sensor surface has an important role in the development of an immunoassay (Altintas et al., 2011). Therefore, different

concentrations of capture antibody (10-100 μg mL^{-1}) were investigated on the sensor surface using standard bacterial cells (10^9 CFU mL^{-1}). **Fig.1**a, show the frequency changes achieved with increasing the polyclonal antibody concentrations immobilisation on the QCM sensor chip, while **Fig.1**b show the binding response of 10^9 CFU mL^{-1} *C. jejuni* bacterial cells at each polyclonal antibody concentration used. From these results, the polyclonal antibody concentration of 70 μg mL^{-1} gave the highest signal responses for both antibody immobilisation and bacterial capture with 560 Hz and 60 Hz, respectively. Higher concentrations of polyclonal antibodies between 70 and 100 μg mL^{-1} caused no further increase in signal, and the immobilisation level and analyte binding started to decrease due to steric hindrance (Buchatip et al., 2010). Thus, the polyclonal antibody concentration of 70 μg mL^{-1} was used in further sensor work.

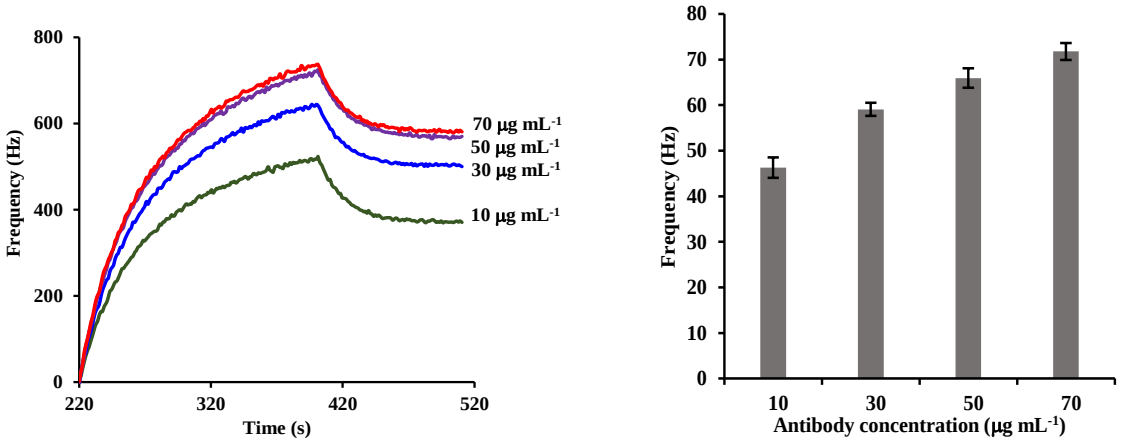
Parallel optimisation tests were performed with monoclonal antibody concentrations (10-70 μg mL^{-1}) as the capture for immobilisation on the sensor chip and the results are reported in **Fig.1**c and d. **Fig.1**c, show the frequency changes achieved with increasing the monoclonal antibody concentrations while **Fig.1**d show the binding response of 10^9 CFU mL^{-1} bacterial cells at each monoclonal antibody concentrations used. From these results, the monoclonal antibody concentration of 50 and 70 μg mL^{-1} gave almost similar sensor responses for both antibody immobilisation and bacterial capture with 578 and 580 Hz, and 65 and 70 Hz, respectively. Due to the similar outputs, 50 μg mL^{-1} of monoclonal antibody can be chosen for future studies because of economical reason. Similar range of optimal concentrations of antibody (50-100 μg mL^{-1}) for immobilisation was also reported in the literature. For instance, Szalontai et al. (2012) reported the use of 50 μg mL^{-1} as the optimal concentration of polyclonal antibody immobilisation to a gold QCM sensor for the detection of *Bifidobacterium bifidum* and *L. acidophilus* while Buchatip et al. (2010) reported 100 μg mL^{-1} as the optimal concentration for immobilising monoclonal antibody raised against *Vibrio harveyi*. The use of higher concentrations of antibody are not frequently reported as high concentrations of antibody could also promote non-specific adsorption (Buchatip et al., 2010) and increase the cost of the test.

Further investigation using 3D images of AFM were carried out to provide evidence for the presence of antibody and bacterial binding to the gold surface. The advent of AFM is to allow further characterisation of the sensor surface at molecular resolution. In the case of

antibody binding to the sensor surface, the quality and quantity of succesful binding can determine the sensitivity of the assay and these factors can be observed under AFM. Hill is the terminology used in describing AFM surfaces variability (Kleo et al., 2012). The topography image on an area of 20 μm and example of roughness profile are shown in **Fig.1**e-g. The bare gold electrode in **Fig.1**e shows a few numbers of hills as it represent the control before the deposition of SAM. The 3D AFM image shows two distinctive types of hills on the surface of the gold-coated glass. The first lower hills are homogenous and relatively dense likely resulting from successful amine groups formation on the SAM surface through EDC and NHS (**Fig.1**f) whilst the second distinctly higher hills were the results of bacteria binding on the polyclonal antibody immobilised on the gold surface (**Fig.1**g). The results obtained in this work indicated that the observed frequency changes seen on the QCM instrument was due to the succesful immobilisations of antibody and bacteria detection on the gold sensor.



(c) **(d)**



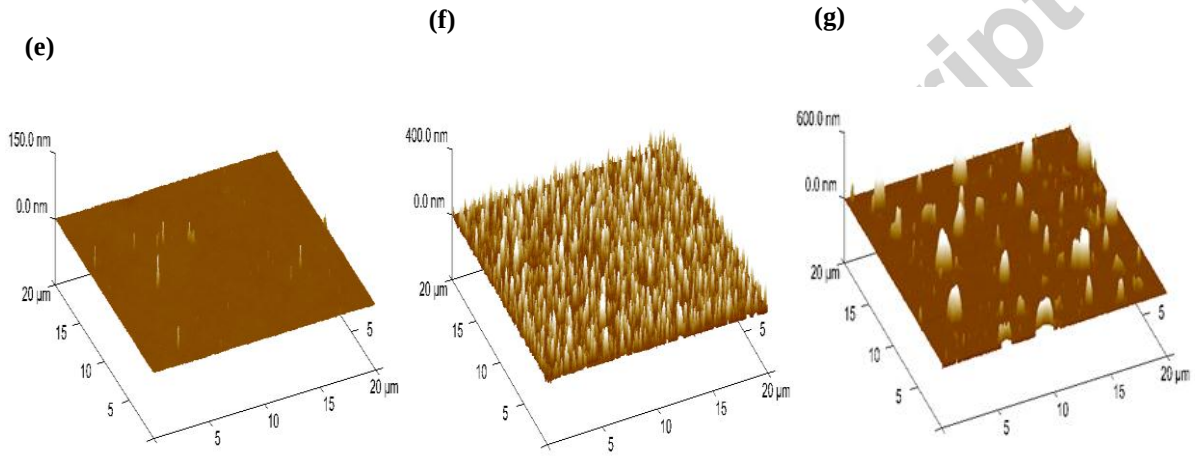


Fig. 1: Sensorgram for optimisation of antibody immobilisation using different concentration of polyclonal antibody (a) and monoclonal antibody (c) against *C. jejuni* at 10^9 CFU mL^{-1} with their resulting frequency responses by utilising polyclonal (b) and monoclonal (d) antibodies. AFM images of bare gold (e), antibody immobilised (f) and binding of 10^5 CFU mL^{-1} *C. jejuni* (g). The AFM topographic image shown is for a 20 µm×20 µm area.

3.2. Optimisation of *Campylobacter jejuni* binding assay

C. jejuni cells were detected by conducting three different assay formats which were direct, sandwich and signal amplification using gold-nanoparticles (AuNPs) conjugated detector antibodies.

3.2.1. Direct assay

In this assay format the *C. jejuni* cells were injected onto the polyclonal or monoclonal antibodies immobilised surface as the capture antibodies. The real-time sensorgram of the direct assay using rabbit polyclonal antibody as capture antibody led to obtain gradual increase on the frequency by increasing cell concentration (**Fig.2**a). A sigmoidal relationship between -df (Hz) (active signal after subtracting the background signal) and *C. jejuni* cells concentration was found in the range of 10^4 CFU mL^{-1} to 10^9 CFU mL^{-1} with the linear portion spanning the bacterial concentrations between 10^5 CFU mL^{-1} to 10^7 CFU mL^{-1} (**Fig.2** b). The LOD at 2.0 x 10^5 CFU mL^{-1} was calculated using the four-parameter logistics equation. The resulting curve had a correlation coefficient of 0.995. The direct assay was also conducted using the mouse monoclonal antibody against *C. jejuni* with same investigation range and a sigmoidal relationship between -df (Hz) and *C. jejuni* cells concentration was found in the range of 10^4 CFU mL^{-1} - 10^9 CFU mL^{-1} with the linear portion spanning the bacterial concentrations of between 10^6 CFU mL^{-1} to 10^8 CFU mL^{-1}. The LOD was at 2.0 x 10^6 CFU mL^{-1} (**Fig.2**c). The resulting curve gave a correlation coefficient of 0.967. Evidently, the monoclonal antibody gave lower response and LOD value compared to polyclonal antibody format in the direct assay.

Based on these results, monoclonal antibody was not used as the capture antibody for sandwich and AuNPs amplification assays, but its usage as the secondary antibody was explored. Direct assay on its own rarely give good sensitivity with label free technology such as QCM biosensor. Ideally, direct assay is the best format for rapid and simple assay development in immunosensor as there is no extra label additions compared to other formats. Some of the recently reported detection limits achieved for microbial detection using direct assay are listed in Supplementary document (**Table S1**). It appears that polyclonal antibody preparations for the detection of bacteria form the majority of the most sensitive direct assay. Apparently, commercially available polyclonal antibody preparation for *E. coli* has long been known to be amongst the most sensitive or has high avidity, and hence used as a benchmark standard to assay the avidity of several direct assay formats for pathogen detection, for instance in the detection of *L. monocytogenes* (Sharma and Mutharasan, 2013). Wei et al. (2007) used four commercial polyclonal antibodies against *Campylobacter* to develop an SPR based biosensor using direct assay achieving 10^3 CFU mL^{-1}. Huang et al. (2010) used monoclonal antibodies immobilised on O-carboxymethylchitosan surface modified Fe_30_4 nanoparticles for the detection of *Campylobacter jejuni*. By employing electrochemical impedance spectroscopy they achieved a detection range of 1.0 x 10^3 to 1.0 x 10^7 CFU mL^{-1}.

Viswanathan et al. (2012) developed a multiplex electrochemical immunosensor for detection of food-borne pathogens. The authors used nanocrystal conjugated antibody with multiwall carbon nanotube-polyallylamine modified screen printed electrode. In their work, a detection limit of 400 cells mL^{-1} *Campylobacter* were achieved using monoclonal antibodies.

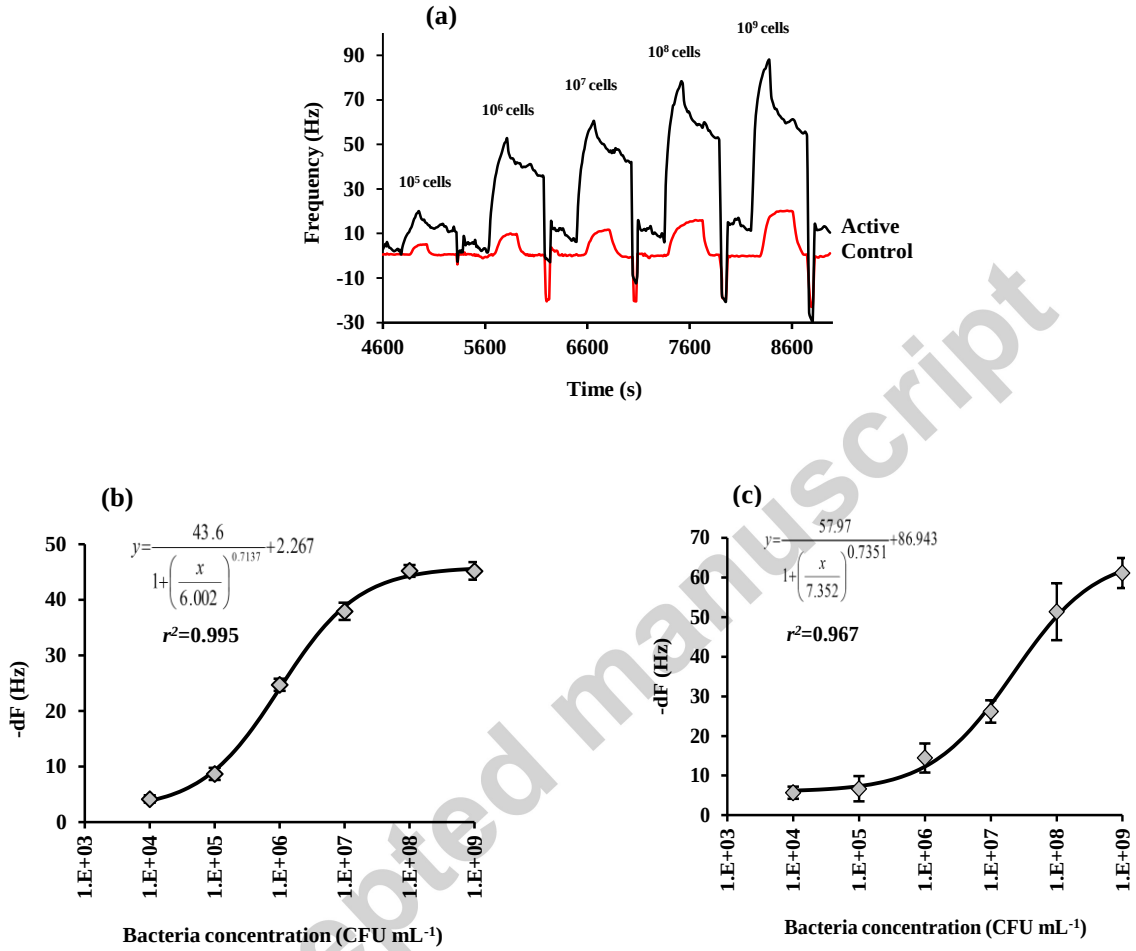


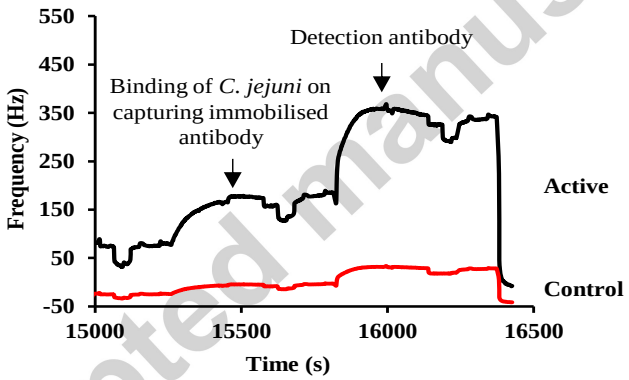
Fig. 2: Frequency response profiles for *C. jejuni* through direct binding assay using polyclonal antibody as capture antibody at different concentrations of *C. jejuni* cells (a), *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration (CFU mL^{-1}) on the QCMA-1 sensor through direct format assay with polyclonal antibody as the capture antibody (b), *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration (CFU mL^{-1}) on the QCMA-1 sensor through direct format assay with monoclonal antibody as the capture antibody (c. Error bars represent average ± standard deviation of triplicates.

3.2.2. Sandwich assay

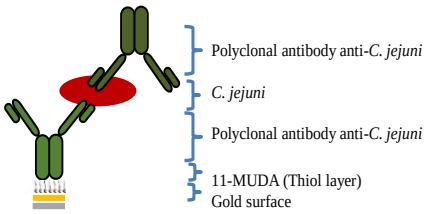
Rabbit polyclonal antibody against *C. jejuni* was used as the primary capture antibody in this assay. The sandwich format not only can improve sensitivity but also has the added benefit to reduce false negative results (Sharma and Mutharasan, 2013). Two different types of antibody as the detection antibody layer were used as shown through schematic diagram in **Fig.3**. The concentration of detection antibody used was 70 μg mL^{-1} for both polyclonal and monoclonal antibody. The results in **Fig.3**a show the real-time sensorgram of sandwich assay by using polyclonal antibody as the capture and detection antibody. A sigmoidal relationship between -df (Hz) and *C. jejuni* cells concentration was found in the range of 10^4 CFU mL^{-1} to 10^8 CFU mL^{-1} with the linear portion spanning the bacterial concentrations of between 10^4 CFU mL^{-1} to 10^7 CFU mL^{-1} (**Fig.3**b). The LOD was calculated as 2.4 x 10^4 CFU mL^{-1}. The resulting curve had a correlation coefficient of 0.998. Results in **Fig.3**c, show a sigmoidal relationship between -df (Hz) and *C. jejuni* cells concentration was found in the range of 10^4 CFU mL^{-1} to 10^8 CFU mL^{-1} with the linear portion spanning the bacterial concentrations of between 10^5 CFU mL^{-1} to 10^8 CFU mL^{-1} through sandwich binding assay with monoclonal antibody as the detection antibody. The LOD was at 3.4 x 10^5 CFU mL^{-1}. The resulting curve had a correlation coefficient of 0.981.

Although the sensitivity was increased when polyclonal antibody was used instead of monoclonal as the detection antibody, this additional binding steps did not allow for the detection of *C. jejuni* at concentrations below 10^4 CFU mL^{-1}. The results also showed that the sandwich assay that utilised monoclonal antibody as the detection antibody had lower performance with LOD at 10^5 CFU mL^{-1} compared to 10^4 CFU mL^{-1} for a polyclonal antibody as the detection antibody. Published works on sandwich assays using QCM have shown significant improvement in terms of LOD values compared to direct assays with more than several orders of magnitude improvement over direct assay. For instance, an increase in sensitivity corresponding to a lowering of LOD values from between 10^3-10^4 CFU mL^{-1} to approximately 10^2 CFU mL^{-1} was reported for the detection of *E. coli* O157:H7 (Shen et al, 2012; 2011), *Salmonella* Typhimurium (Salam et al., 2013) and *Listeria monocytogenes* (Sharma and Mutharasan, 2013).

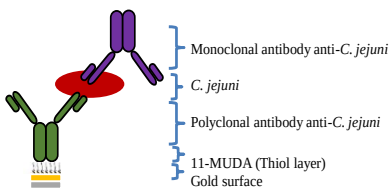
(a)



(b)



(c)



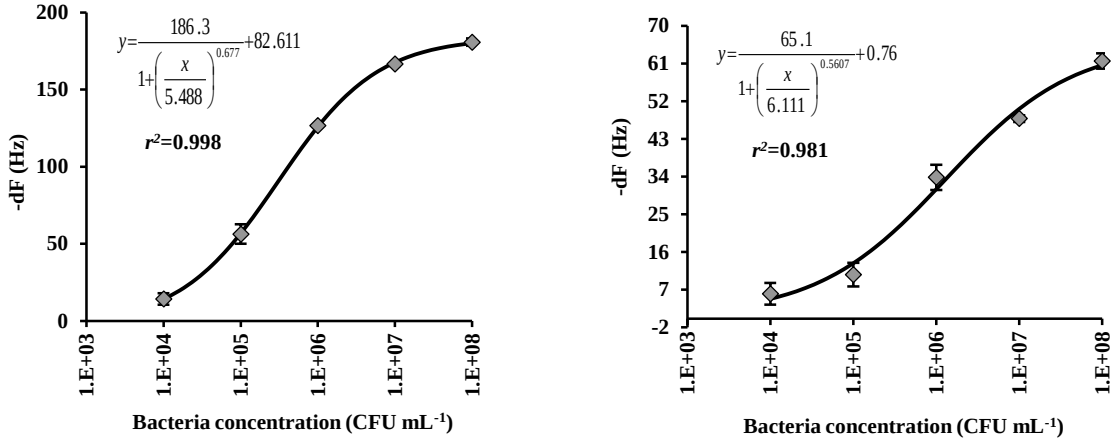


Fig. 3: Frequency response profiles for *C. jejuni* through sandwich binding assay with polyclonal antibody as detection antibody (a), *C. jejuni* standard plot with -dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich format assay with polyclonal antibody as the detection antibody (b) and *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich format assay with monoclonal antibody as the detection antibody (c). Error bars represent average ± standard deviation of triplicates.

3.2.3. Sandwich assay using AuNPs for signal amplification

To enhance the sensitivity of the QCMA-1 immunosensor device, a signal enhancement method was developed by employing the application of AuNPs conjugated with antibody as the detection layer after injection of antigen to increase the mass bound to the surface. For this work, two different types of antibody, rabbit polyclonal and mouse monoclonal conjugated with AuNPs were injected on the polyclonal antibody immobilised surface as the detection antibody layer (**Fig.4)**. The primary capturing antibody used was the rabbit polyclonal antibody against *C. jejuni*. A 20x dilution of the antibody-conjugated AuNPs stock in PBS was used in the entire *C. jejuni* binding assay for both polyclonal and monoclonal antibodies. Both antibody-conjugated AuNPs were used as the detection layer after the injection of *C. jejuni* cells. The time of the assay for each *C. jejuni* concentration was about 9 min. In the sandwich assay with polyclonal antibody-conjugated AuNPs, a near-sigmoidal relationship between -df (Hz) and *C. jejuni* cells concentration was found in the range of 10^1 CFU mL^{-1} to 10^6 CFU mL^{-1} with the linear portion spanning the bacterial concentrations of between 10^3 CFU mL^{-1} and 10^5 CFU mL^{-1} (**Fig.5a**). The LOD was at 1.5 x 10^2 CFU mL^{-1}. The resulting curve had a correlation coefficient of 0.994. In the sandwich assay with monoclonal antibody-conjugated AuNPs, a near-sigmoidal relationship between *C. jejuni*

cells concentration was found in the range of 10^2 CFU mL^{-1} to 10^5 CFU mL^{-1}. As the assay was only conducted up to bacterial concentrations of 10^5 CFU mL^{-1} (**Fig.4b**), the linear regression curve is not extended above this concentration.. The LOD was calculated at 6 x 10^4 CFU mL^{-1}. The resulting curve had a correlation coefficient of 0.961.

Table 1 gives the summary of the developed assays in this work. The use of nanoparticles in sensors has risen over the past years due to their good biocompatibility. Due to this, they have been used to immobilise biomolecules such as DNA, carbohydrate, proteins and antibody for the fabrication of biosensors (Srivastava et al., 2014). Nanoparticles display unique effects such as mini size effect, surface effect, quantum size effect and macro-quantum tunnel effect (Hao et al., 2011; Altintas et al., 2014) that are useful in sensor works. Early research on the use of nanoparticle on QCM sensors for pathogen detection utilises DNA as the principal ligands (Kaewphinit et al., 2012; Hao et al., 2011; Wang et al., 2008; and Mao et al., 2006). Only recently nanoparticles such as AuNPs (Salam et al., 2013; Reddy et al., 2012; Guo et al., 2012 and Shen et al., 2011) and silver (Srivastava et al., 2014) have been utilised in QCM-based sensors with antibody as the ligand.

Other works on the use of antibody conjugated AuNPs in sandwich assay for pathogen using QCM also shows vast improvement of LOD. For instance, Guo et al. (2012) reported one log improvement for *E. coli* O157:H7 detection on a QCM instrument when AuNPs amplification was added to the sandwich assay format. Kleo et al. (2012) reported an improvement of LOD from 10^5 - 10^6 to 10^3 when AuNPs functionalised antibody in a sandwich assay was used in the determination of *Francisella tularensis*. Shen et al. (2011) reported an LOD value of 53 CFU mL^{-1} for the detection of *E. coli* O157:H7 while Salam et al. (2013) reported an LOD value of 23 CFU mL^{-1} in detecting *Salmonella* Typhimurium, all in sandwich assay format.

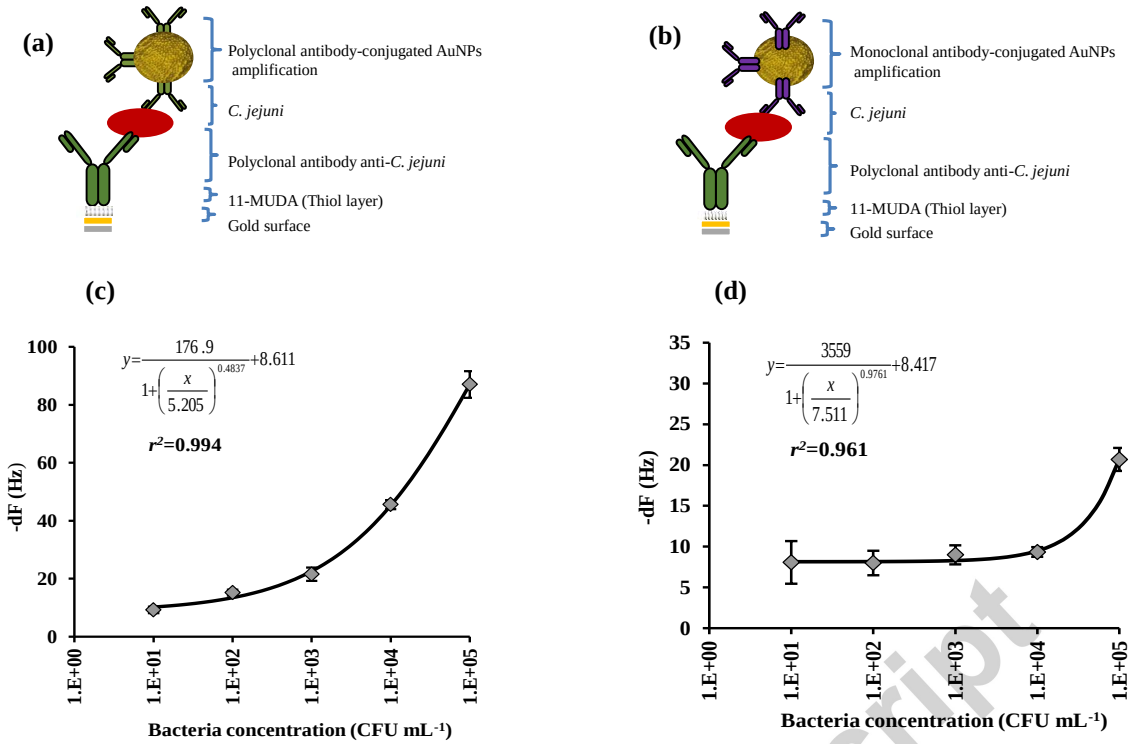


Fig. 4: *C. jejuni* standard plot with -dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich assay with polyclonal antibody-conjugated AuNPs as detection antibody (a) and *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich assay with monoclonal antibody-conjugated AuNPs as detection antibody (b). Error bars represent average ± standard deviation of triplicates.

Table 1: Summary of the *C. jejuni* detection sensor results.

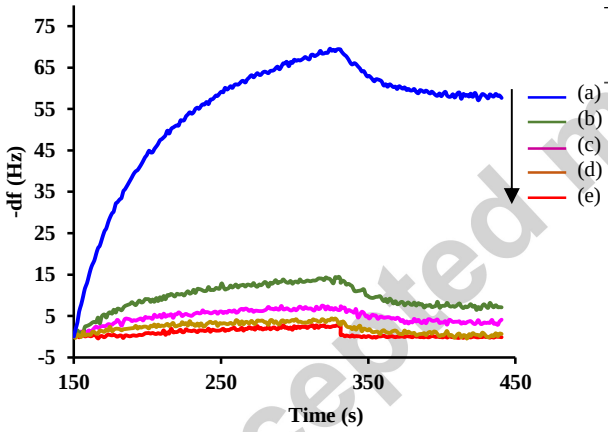
Assay type	Capture antibody	Detector antibody	Regeneration	Investigation range (CFU mL^{-1})	LOD (CFU mL^{-1})
Direct 1	Polyclonal Ab	-	Yes	10^4-10^9	2.0 x 10^5
Direct 2	Monoclonal Ab	-	Yes	10^4-10^9	2.0 x 10^6
Sandwich 1	Polyclonal Ab	Polyclonal Ab	Yes	10^4-10^8	2.4 x 10^4
Sandwich 2	Polyclonal Ab	Monoclonal Ab	Yes	10^4-10^8	3.4 x 10^5
Signal Amp. 1	Polyclonal Ab	Polyclonal Ab	Yes	10-10^5	1.5 x 10^2
Signal Amp. 2	Polyclonal Ab	Monoclonal Ab	Yes	10-10^5	6.0 x 10^4

*****Amp: Amplification; Ab: Antibody

3.3. QCM immunosensor selectivity against other bacteria

Cross reactivity studies is an important parameter which reflect the specificity and realibility of the developed immunosensor. Antibodies can react with other substances or other bacterial contaminate in the food matrix. In order to study the specificity of the QCM sensor to *C. jejuni*, the bacterium was replaced by the other foodborne pathogens (*S.* Typhimurium, *L. monocytogenes* and *E. coli*) at the concentration of 10^5 CFU mL^{-1}.

A very low cross reactivity (<10%) was observed with *Salmonella* Typhimurium and *L. monocytogenes*, while there was no cross reactivity towards *E. coli* (**Fig.5**). All of the bacteria used in this work are foodborne pathogen and are often present together with *C. jejuni*. The results obtained showed that the developed immunosensor based on QCM is very specific to *C. jejuni* and showed minimal cross reactivity to other foodborne pathogen indicating the suitability of the developed sensor to be used for *C. jejuni* biomonitoring work.



Bacteria tested (10^4 CFU mL^{-1})	Response (-dF, Hz)	QCM immunosensor % relative-activity
(a) *Campylobacter jejuni*	58.32	100±2.31
(b) *Salmonella* Typhimurium	7.15	7.15±2.08
(c) *Listeria monocytogenes*	3.09	4.25±1.03
(d) *Escherichia coli*	0.00	0.00±0.14
(e) Control	0.00	0.00±0.04

Fig. 5: Frequency response profile for *C. jejuni* binding (a) compared with other foodborne bacteria, *Salmonella* Typhimurium (b), *Listeria monocytogenes* (c), *Escherichia coli* (d) and control (e) tested using the sandwich assay with polyclonal-conjugated AuNPs. The concentration of all bacteria tested was at 10^5 CFU mL^{-1} and the percentage of cross reactivity is shown on the right.

4. Conclusions

This work describes the successful development of a QCM immunosensor for the sensitive, rapid and specific detection of the foodborne pathogen *C. jejuni*. A detection limit of 150 CFU mL^{-1} was achieved using the sandwich assay by utilising AuNPs conjugated detection

antibody. The signal amplification of AuNPs and improvement of the sensitivity over direct detection method demonstrated that nanoparticles could be employed to enhance the sensitivity of QCM immunosensors for the detection of foodborne *C. jejuni*. This comprehensive study highlights the great potential of the developed sensor to be used for food samples analysis and developing sample pretreatments methods.

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Legends of Figures

Figure 1: Sensorgram for optimisation of antibody immobilisation using different concentration of polyclonal antibody (a) and monoclonal antibody (c) against *C. jejuni* at 10^9 CFU mL^{-1} with their resulting frequency responses by utilising polyclonal (b) and monoclonal (d) antibodies. AFM images of bare gold (e), antibody immobilised (f) and binding of 10^5 CFU mL^{-1} *C. jejuni* (g). The AFM topographic image shown is for a 20 μm×20 μm area.

Figure 2: Frequency response profiles for *C. jejuni* through direct binding assay using polyclonal antibody as capture antibody at different concentrations of *C. jejuni* cells (a), *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration (CFU mL^{-1}) on the QCMA-1 sensor through direct format assay with polyclonal antibody as the capture antibody (b), *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration (CFU mL^{-1}) on the QCMA-1 sensor through direct format assay with monoclonal antibody as the capture antibody (c. Error bars represent average ± standard deviation of triplicates.

Figure 3: Frequency response profiles for *C. jejuni* through sandwich binding assay with polyclonal antibody as detection antibody (a), *C. jejuni* standard plot with -dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich format assay with polyclonal antibody as the detection antibody (b) and *C. jejuni* standard plot with –dF (Hz) versus *C. jejuni* concentration on the QCMA-1 sensor through sandwich format assay with monoclonal antibody as the detection antibody (c). Error bars represent average ± standard deviation of triplicates.

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Legends of Tables

Table 1: Comparision of the *C. jejuni* detection assay results.

List of Figures:

Figure 1

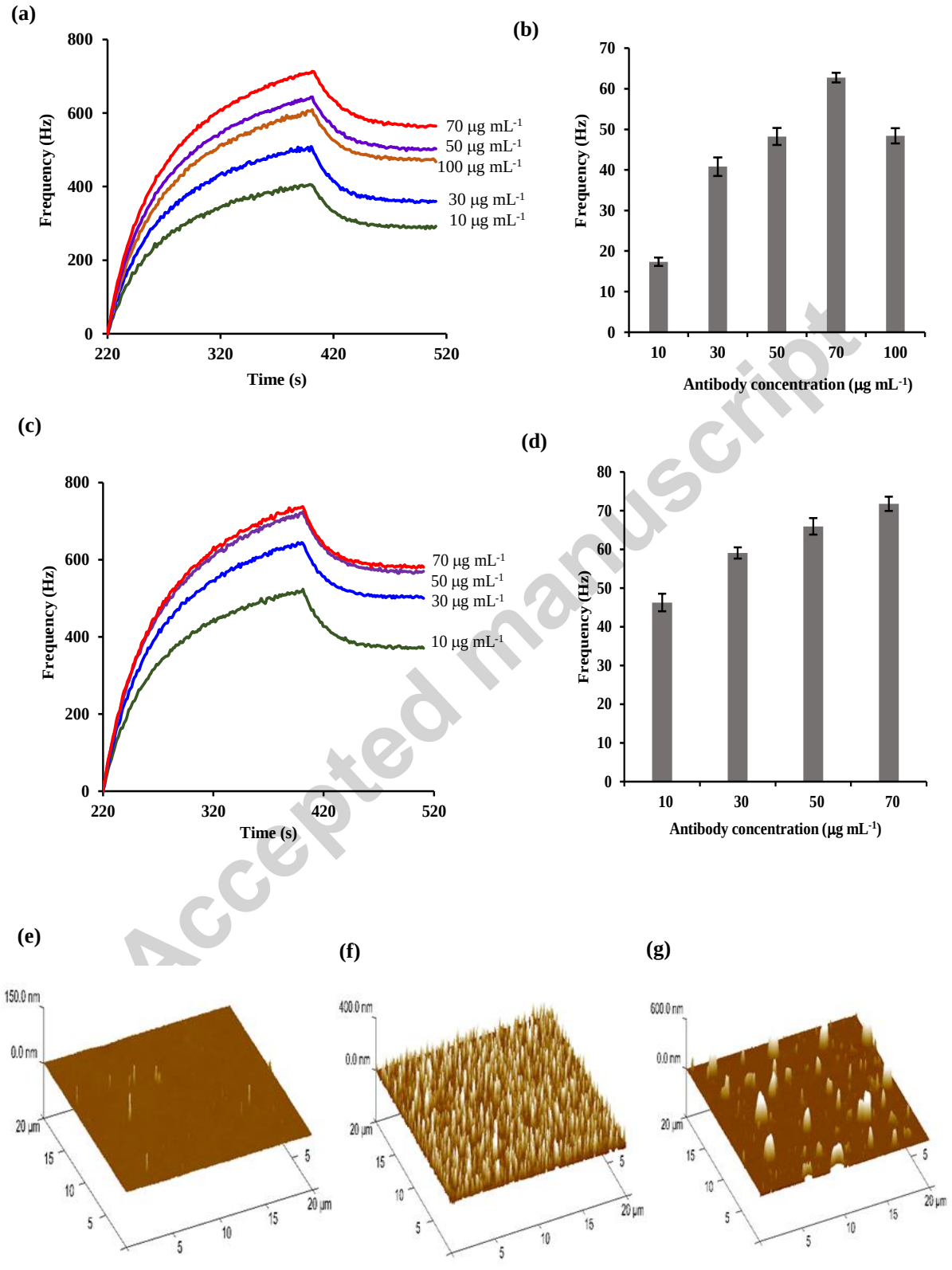
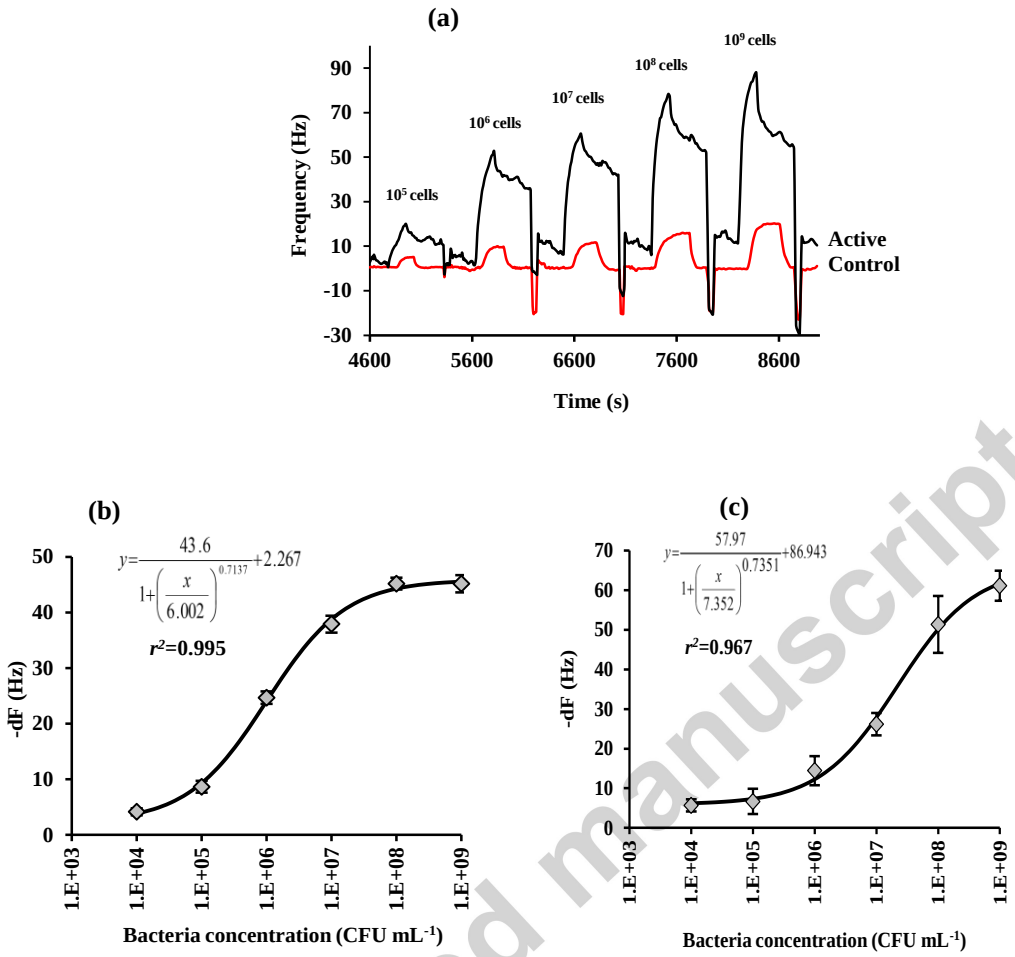


Figure 2



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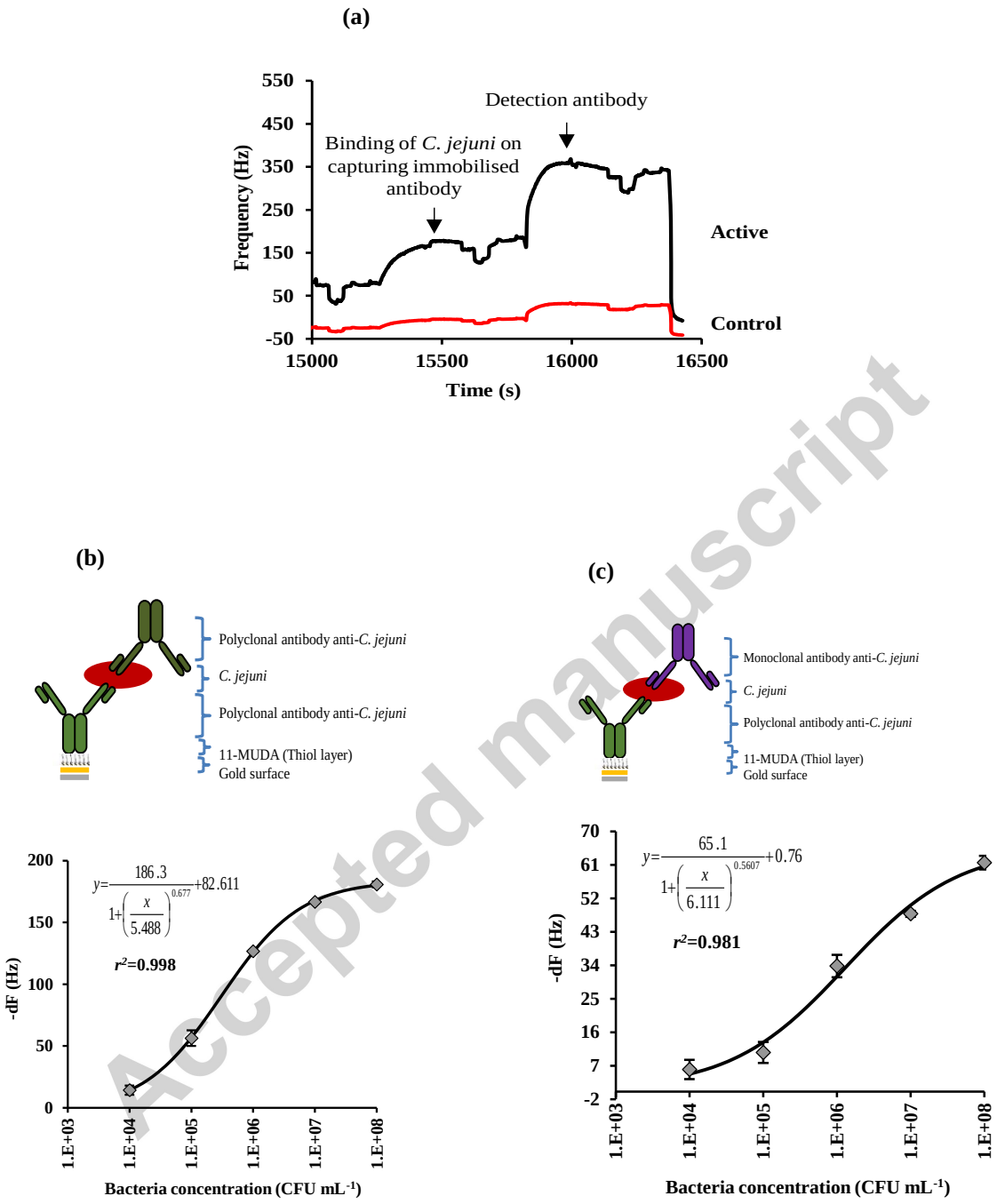

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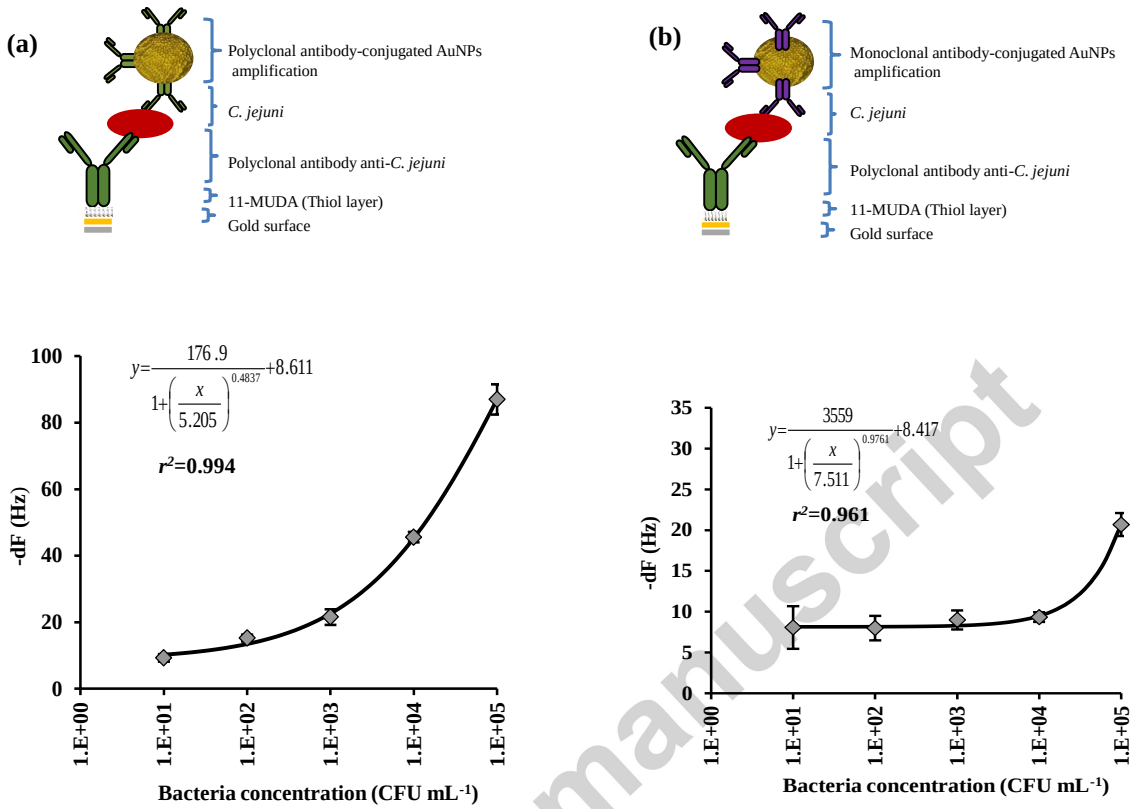

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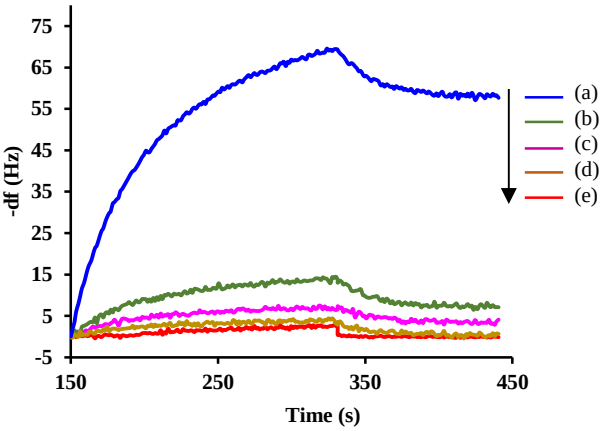

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Table 1:

Assay type	Capture antibody	Detector antibody	Regeneration	Investigation range (CFU mL^{-1})	LOD (CFU mL^{-1})
Direct 1	Polyclonal Ab	-	Yes	10^4-10^9	2.0 x 10^5
Direct 2	Monoclonal Ab	-	Yes	10^4-10^9	2.0 x 10^6
Sandwich 1	Polyclonal Ab	Polyclonal Ab	Yes	10^4-10^8	2.4 x 10^4
Sandwich 2	Polyclonal Ab	Monoclonal Ab	Yes	10^4-10^8	3.4 x 10^5
Signal Amp. 1	Polyclonal Ab	Polyclonal Ab	Yes	10-10^5	1.5 x 10^2
Signal Amp. 2	Polyclonal Ab	Monoclonal Ab	Yes	10-10^5	6.0 x 10^4

*****Amp: Amplification; Ab: Antibody

Highlights

- QCM sensor for *Campylobacter* detection has been developed.
- A fully automated sensor system can play a major role in diagnosing foodborne pathogens.
- Development of highly specific and sensitive biosensor platform can aid in early detection of *Campylobacter jejuni* .
- Amplification of sensor signal with nanoparticles achieved high sensitivity.

- The technology provides reliable and cost-effective sensing tool.