Biosensors and Bioelectronics 59 (2014) 342–349

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

# Automated microfluidically controlled electrochemical biosensor for the rapid and highly sensitive detection of *Francisella tularensis*

Samuel B. Dulay [a,*], Rainer Gransee [b], Sandra Julich [c], Herbert Tomaso [c], Ciara K. O' Sullivan [a,d,**]

[a] Nanobiotechnology and Bioanalysis Group, INTERFIBIO, Departament d'Enginyeria Quimica, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona, Spain
[b] Fraunhofer ICT-IMM, Carl-Zeiss-Strasse 18-20, 55129 Mainz, Germany
[c] Institute of Bacterial Infections and Zoonoses, Friedrich-Loeffler-Institut, Naumburger Strasse 96 a, Jena 07743, Germany
[d] Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys, 23, 08010 Barcelona, Spain

ARTICLE INFO

*Article history:*
Received 5 February 2014
Received in revised form
8 March 2014
Accepted 11 March 2014
Available online 12 April 2014

*Keywords:*
Electrochemical immunosensors
*Francisella tularensis*
Self-assembled monolayer (SAM)
Lab-on-a-chip

ABSTRACT

Tularemia is a highly infectious zoonotic disease caused by a Gram-negative coccoid rod bacterium, *Francisella tularensis*. Tularemia is considered as a life-threatening potential biological warfare agent due to its high virulence, transmission, mortality and simplicity of cultivation. In the work reported here, different electrochemical immunosensor formats for the detection of whole *F. tularensis* bacteria were developed and their performance compared. An anti-*Francisella* antibody (FB11) was used for the detection that recognises the lipopolysaccharide found in the outer membrane of the bacteria. In the first approach, gold-supported self-assembled monolayers of a carboxyl terminated bipodal alkanethiol were used to covalently cross-link with the FB11 antibody. In an alternative second approach F(ab) fragments of the FB11 antibody were generated and directly chemisorbed onto the gold electrode surface. The second approach resulted in an increased capture efficiency and higher sensitivity. Detection limits of 4.5 ng/mL for the lipopolysaccharide antigen and 31 bacteria/mL for the *F. tularensis* bacteria were achieved. Having demonstrated the functionality of the immunosensor, an electrode array was functionalised with the antibody fragment and integrated with microfluidics and housed in a tester set-up that facilitated complete automation of the assay. The only end-user intervention is sample addition, requiring less than one-minute hands-on time. The use of the automated microfluidic set-up not only required much lower reagent volumes but also the required incubation time was considerably reduced and a notable increase of 3-fold in assay sensitivity was achieved with a total assay time from sample addition to read-out of less than 20 min.



## 1. Introduction

In the past decades, there has been increased interest in developing new methods for the rapid detection of microorganisms including toxigenic and pathogenic organisms (Grunow et al., 2000; Magnarelli et al., 2007; Savitt et al., 2009). Tularemia, also known as rabbit fever, is a highly infectious zoonotic disease caused by the non-motile, non-spore-forming, Gram-negative coccoid rod bacterium, *Francisella tularensis*. It occurs naturally in lagomorphs (rabbits and hares), but many animals have been reported to be infected. Transmission to humans is mostly associated with inhalation of aerosolised bacteria, handling of infected animals, arthropod bites, and ingestion of contaminated foods and water (Kleo et al., 2012; Magnarelli et al., 2007). The usual incubation period is 3–5 days but symptoms can become visible between 1 and 21 days following exposure depending on the route of infection. Clinical manifestation of the disease in humans can occur in different forms ranging from skin ulcers to more severe forms such as life threatening-pneumonia (Penn, 2005).

Despite the fact that most of tularemia infections can be treated with antibiotics (Dennis et al., 2001), it is still considered as life-threatening due to its high virulence, transmission and mortality (Su et al., 2007). *F. tularensis* could be used as a potential biological warfare agent or in terrorist attacks. Identification of *F. tularensis* has been achieved using cultivation and molecular techniques including polymerase chain reaction (PCR) (Johansson et al., 2000) and real time PCR assays (Bystrom et al., 2005; Simşek et al., 2012; Versage et al., 2003). Besides the detection of the bacterial cell, the

* Corresponding author.
** Corresponding author at: Nanobiotechnology and Bioanalysis Group, INTERFIBIO, Departament d'Enginyeria Quimica, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona, Spain.
*E-mail addresses:* samuelbacena.dulay@estudiants.urv.cat (S.B. Dulay), ciara.osullivan@urv.cat (C.K. O' Sullivan).

http://dx.doi.org/10.1016/j.bios.2014.03.024
0956-5663/

detection of specific antibodies in serum is the most widely used serological analysis technique for routine laboratory diagnosis of tularemia (Porsch-Ozcürümez et al., 2004). Enzyme-linked immunosorbent assay (ELISA) (Pohanka and Skládal, 2008), Western blot and other immunological assays can be used to detect seroconversion in patients. However, antibodies to *F. tularensis* appear only 2 weeks or more after infection (Emanuel et al., 2003). An early identification of the pathogen is important and serological diagnosis is not useful as a rapid diagnostic approach (Grunow et al., 2000). Although these standard techniques are sensitive, they are rather time consuming and require intensive hands-on-time.

The detection of *F. tularensis* is required in different situations which include civil rescue and security units, homeland security, military operations, public transportation securities such as airports, metro and railway stations due to its harmful effect on the human population (Skládal et al., 2010). Therefore, there is a need for a specific detection system which could be portable, rapid and cost-effective.

Electrochemical biosensors are renowned for their excellent sensitivity, selectivity, versatility, and simplicity, (Lazcka et al., 2007; Pividori et al., 2009). There is a continual interest in the development of these technologies for application in clinical diagnostics, food quality control and environmental monitoring (Warsinke et al., 2000), as promising alternatives to traditional methods in detecting pathogens (Pohanka et al., 2007). Many of the electrochemical immunosensor format architectures try to mimic that found in standard ELISA where different strategies in immobilisation of the biocomponent on the transducer's surface have been developed. The use of self-assembled monolayers (SAM) has garnered increased interest due to the possibility of avoiding random orientation of the biocomponents (antibody/antigen) on the surface, good surface coverage and the possibility of incorporating efficient surface protection moieties to eliminate non-specific binding. Specifically targeting *F. tularensis*, Kleo et al. (2012) successfully demonstrated the immobilisation of an antibody using a carboxy-terminated thiol layer onto a gold surface and *F. tularensis* bacterial cells were directly detected by frequency change using quartz crystal microbalance technique, achieving a detection limit of $4 \times 10^3$ CFU/mL. Skladal et al. (2005) used a sandwich format for the amperometric detection of inactivated *F. tularensis* bacterial cells obtaining a detection limit of 100 CFU/mL in 30 min.

In this work, we report a biosensor for the detection of whole inactivated *Francisella tularensis* live vaccine strain (LVS) bacterial cells comparing the use of whole antibodies immobilised via chemical cross-linking to a bipodal alkyl thiol self-assembled monolayer (SAM) and a SAM formed from the direct chemisorption of F(ab) antibody fragments through their free sulfhydryl group which inherently correctly orients the antibody. The biosensor was housed within a microfluidic set-up and the assay was automated via the use of a peristaltic pump, with the only required end-user intervention being sample addition. Parameters such as incubation time and temperature were optimised and applied to the detection of both the lipopolysaccharide (LPS) antigen and whole *F. tularensis* bacterial cells.

## 2. Experimental

All the starting materials were obtained from commercial suppliers and used without further purification. Monoclonal antibodies (mAb) FB11 and T14 were purchased from Hytest Ltd. Company, Finland. Lipopolysaccharide (LPS) from *Francisella tularensis* was purchased from MICROMUN Privates Institut für Mikrobiologische Forschung GmbH, Germany. Whole cell bacteria of *Francisella tularensis* LVS subsp. *holarctica*, *F. tularensis* subsp. *novicida*, *Yersinia enterocolitica* subsp. *enterocolitica*, and *Yersinia pseudotuberculosis* were kindly provided by the Friedrich-Loeffler-Institut, Institut für bakterielle Infektionen und Zoonosen, Germany. 1-(mercaptoundec-11-yl)tetra (ethyleneglycol) (PEG) was purchased from Sigma-Aldrich Co., sulfuric acid, strontium nitrate, phosphate-buffered saline (PBS) (dry powder), PBS-Tween-20, hydrogen peroxide 30% v/v, ethanolamine, acetone and ethanol (synthetic grade), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), and acetic acid were purchased from Scharlau (Spain); 22-(3,5-bis((6 mercaptohexyl)oxy) phenyl)-3,6,9,12,15,18,21-heptaoxadocosanoic acid dithiol PEG-6 carboxylate (DT2) was purchased from SensoPath Technologies (USA) and 3,3,5,5-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA was obtained from Sigma. Aqueous solutions were prepared with Milli-Q water Millipore (18 mΩ cm) and all reagents were used as received.

### 2.1. Instrumentation

Electrochemical studies were carried out using an Autolab PGSTAT 10 potentiostat with measurements performed using either a conventional three-electrode cell for 3 mm-diameter lithographically produced gold electrodes (for developmental work) or 1 mm-diameter electrodes. An array of 24 gold electrodes with internal reference and counter electrodes was used for the final format with the biosensor integrated within the microfluidic set-up. The lithographically produced gold electrodes were provided by Fraunhofer ICT-IMM (IMM), Germany, and were produced as previously reported (Fragoso et al., 2011). In the developmental work, a standard silver/sliver chloride (sat. KCl) was used as a reference electrode (CHI 111 CH Instruments) and platinum gauze as the counter electrode. All sonication procedures were conducted with an ultrasonic bath (Branson ultrasonic corporation, model 2510E-MT, USA). Enzyme-linked immunosorbent assay (ELISA) studies were performed using bioNOVA cientifica, S.L. (Madrid, Spain) and HydroFlex 3-in-1 well washer, TECAN (Spain).

The microfluidic set-up for incubation of analytes and flushing/washing with built in peristaltic pump was designed together with and provided by IMM, Germany and the polymeric microfluidics were supplied by microfluidic ChipShop GmbH, Germany.

### 2.2. Preparation and characterisation of fragment antibodies

#### 2.2.1. Preparation of $(Fab)_2$ Fragments

Anti-*Francisella tularensis* antibodies (FB11 and T14), were purified using YM100 KDa microcon and washed four times with 0.15 M NaCl. Fifty microlitres of 0.5 M Tris buffer containing 50 mM EDTA (pH 7) was added to 500 μL of the purified antibodies (1 mg/mL), followed by 50 μL (10 mg/mL) of bromelain solution in PBS (pH 7.2, 0.01 M). The mixture was incubated at 37 °C overnight. The produced $F(ab')_2$ fragments were separated with a Sephacryl S-100 column using 0.15 M NaCl as the eluting agent. The fractions with maximum absorbance at 280 nm were concentrated using a YM10 KDa microcon, washed several times with PBS (pH 7.4, 0.01 M), and stored at −20 °C (Nassef et al., 2009).

#### 2.2.2. Preparation and characterization of F(ab) Fragments (F(ab) FB11 and F(ab)T14)

The F(ab) fragments were always prepared freshly from $F(ab)_2$ fragments before use. 1500 μL of 10 mM cysteine in PBS (pH 10.3) was added to 60 μL (0.483 mg/mL) of $F(ab)_2$ fragments. The mixture was incubated for 2 h at room temperature under gentle shaking and the excess of cysteine was removed using a YM10 KDa microcon. The obtained fragments were washed with PBS (pH 7.4, 0.01 M) four times and quantified spectrochemically at 280 nm (Nassef et al., 2009). The fragments were characterised by non-reducing sodium dodecyl

sulphate-polyacryamide gel electrophoresis analysis (SDS-PAGE, 12% acrylamide in tris-glycine gel) (Riddles, 1983). A precision protein standard marker (Bio-Rad, Nazareth Eke, Belgium was used as a molecular weight reference). Coomasie brilliant blue R250 (Sigma, Spain) was used to stain the proteins.

#### 2.2.3. Preparation of whole-antibody and F(ab)-horseradish peroxidase conjugates

Whole antibodies (FB11 and T14) were purified prior to conjugation to eliminate any preservatives present that would affect the efficiency. The purified whole antibodies were dissolved in 0.1 M MES, 0.15 mM NaCl solution at pH 7.2 to a final volume of 1500 μL and subsequently 10 μL of SATA (*N*-Succinimidyl S-Acetylthioacetate) in DMSO was added (15:1 Ab:SATA). The mixture was then gently stirred in a thermomixer for 30 min at room temperature, protected from light. To deprotect the sulphydryl groups formed, 100 μL of 0.5 M hydroxylamine hydrochloride in 0.15 mM NaCl adjusted to pH to 7.2 was added and were reacted for 2 h at room temperature, again protected from light. Maleimide activated horse radish peroxidase (Maleimide HRP) was then added to the previous mixture to a final concentration of 4:1 (Maleimide HRP:Ab ratio) and incubated for 90 min at 37 °C, after which 2-mercapto ethanol was added to a final concentration of 0.0015 M to stop the reaction. The final products were purified using a YM100 KDa cut-off microcon and were washed with buffer and stored at −20 °C.

#### 2.2.4. ELISA evaluation of optimum antibody combination for sandwich assay

Monoclonal Antibody (mAb) FB11 and T14 (10 μg/mL in carbonate buffer, pH 9.5) were prepared separately and added to each well of a NUNC immunosorp microtiter plate and incubated for 30 min at 37 °C. Following thorough washing with PBS-Tween 20 (pH 7.4, 0.01 M), the plate was then blocked by addition of 200 μL of PBS-Tween 20 (pH 7.4, 0.01 M) and incubated for 1 h at 37 °C, followed by thorough washing of the plate. In the immunorecognition step, 50 μL each of a range of concentrations of *F. tularensis* LPS antigen in PBS-Tween 20 (pH 7.4, 0.01 M) were added to each well coated with mAb FB11 or T14. The plate was again incubated, under shaking conditions for 30 min at 37 °C, and subsequently thoroughly washed with PBS-Tween 20, prior to exposure to different concentrations of mAb-HRP conjugates as a secondary labelled antibody, i.e. FB11-HRP and T14-HRP, and again left to incubate under shaking conditions for 30 min at 37 °C. After a final wash, 50 μL of TMB for ELISA substrate was added to each well and product formation allowed to proceed for at least 15 min at room temperature. The reaction was finally stopped by addition of 1 M $H_2SO_4$, and the absorbance read at 450 nm. Analysis was carried out in triplicate.

### 2.3. Cultivation and inactivation of Francisella tularensis bacteria suspension

*Francisella tularensis* LVS was cultivated on cysteine heart agar (Becton Dickinson GmbH, Heidelberg, Germany) supplemented with 10% chocolatized sheep blood. Incubation was carried out for 3 days at 37 °C in an atmosphere with 5% $CO_2$. Heat assisted inactivation was carried out for 10 min at 95 °C (Thermomixer Compact, Eppendorf AG, Hamburg, Germany). To check sterility, the suspension was plated on agar plates and incubated for 7 days and no growth was observed.

### 2.4. Electrode modification and electrochemical detection

Prior to modification of the lithographic gold electrodes, a three-step cleaning protocol was applied. Initially, in order to remove the protective resist used during storage, the electrodes were sonicated for 5 min in acetone (2 times), 5 min in isopropanol (3 times) and rinsed with water and dried with compressed air. In a second step, the electrodes were then further treated with cold Piranha's solution (*Warning: Piranha's solution is highly corrosive and violently reacts with organic materials*; *this solution is potentially explosive and must be used with extreme caution*) for 30 s and subsequently washed with Milli-Q water before use. In the last step, electrochemical cleaning was performed in 0.5 M $H_2SO_4$ by application of a constant potential of 1.6 V for 10 s followed by 10 voltammetric cycles in the potential range −0.2 to 1.6 V at a scan rate of 0.3 V/s. Finally, the electrodes were rinsed with Milli-Q water and dried with nitrogen.

Modification of the cleaned electrodes was carried out in two ways; (1) formation of a SAM of the bipodal alkanethiol DT2 followed by covalent linking of whole anti-*F. tularensis* antibody and (2) direct chemisorption of F(ab) fragments. In the DT2 immobilisation, electrodes were immersed in an ethanolic solution of 1 μM DT2 for 3 h, then rinsed with ethanol and dried with argon. To activate the carboxylic acids of the DT2, the electrodes were then immersed in a mixture 50% (v/v) EDC (0.2 M) and 50% (v/v) NHS (0.05 M) for 30 min and then rinsed with Milli-Q water. Subsequently, 100 μg/mL of monoclonal antibody (mAb) FB11 in PBS (pH 7.4, 0.01 M) was added to the electrodes modified with the activated DT2 for 30 min and then rinsed with Milli-Q water. Finally, any unreacted activated carboxylic acid groups remaining were blocked by immersion of the electrodes for 15 min in ethanolamine pH 8.5, followed by a final rinse with Milli-Q water.

For the immobilisation of F(ab) fragments, the fragments were prepared as previously reported (Nassef et al., 2009). SAM formation of the F(ab) fragments into gold electrode was carried out by overnight immobilisation of 100 μg/mL F(ab) prepared in PBS (pH 7.4, 0.01 M) solution at room temperature. Blocking with 1 mM poly(ethylene)glycol (PEG) was carried out for 30 min, and the

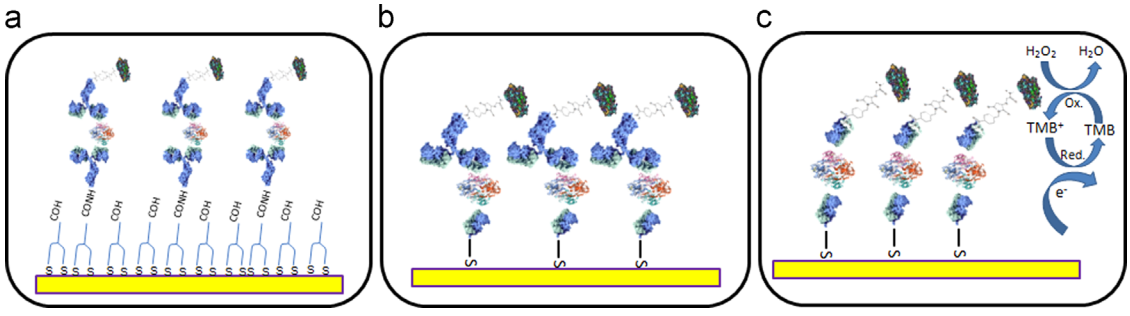



**Fig. 1.** Schematic representation of (a) sandwich with whole antibody linked to bipodal alkyl thiol (DT2) and whole antibody-HRP conjugate (b) sandwich with direct chemisorption of F(ab) fragment and whole antibody-HRP conjugate (c) sandwich with direct chemisorption of F(ab) fragment and F(ab)-HRP conjugate.

modified surfaces were incubated in PBS-Tween-20 (pH 7.4, 0.05 M) for another 30 min to avoid non-specific adsorption.

The electrodes were then exposed to different concentration of LPS or *F. tularensis* LVS in PBS (pH 7.4, 0.01 M) for a specified period of time (2–20 min) and then incubated for a defined period of time (2–20 min) with the corresponding horseradish peroxidase labelled monoclonal antibody. Amperometric measurement was carried out at 0.15 V vs. Ag/AgCl. All the electrochemical measurements were performed at room temperature. The overall immobilization process and detection mechanism can be seen in Fig. 1.

## 3. Results and discussion

### 3.1. ELISA optimisation on assay combination

Monoclonal antibodies (mAb) FB11 and T14 can both recognise and react with the LPS antigen of *Francisella tularensis* and these antibodies were compared in terms of their performance for the detection of the bacteria using ELISA technique. Four different assay constructions combining both antibodies at different concentration conditions of the secondary labelled antibody were evaluated to find the optimum conditions for the best assay format (Supplementary information Fig. S1–S3). It was observed that FB11-LPS–T14HRP and T14-LPS–T14HRP assay combinations gave best results, achieving limits of detection of 10 and 21 ng/mL, respectively. As the FB11-LPS–T14HRP assay format was more sensitive (Supplementary information Fig. S3b), this format was used for the construction of the electrochemical immunosensor.

### 3.2. Preparation and characterisation of fragment antibodies

Whole mAbs (FB11 and T14) were first digested with bromelain to obtain their corresponding $(Fab)_2$ fragment following chromatographic purification. The fragmentation of whole monoclonal antibodies to $(Fab)_2$ fragment and the subsequent chemical reduction using cysteine to F(ab) fragments was successfully achieved. The obtained fragments were characterised using non-reducing gel electrophoresis. The gel characterisation showed bands confirming for the successful generation of fragments from whole antibody (See Supplementary information Fig. S4 for more details).

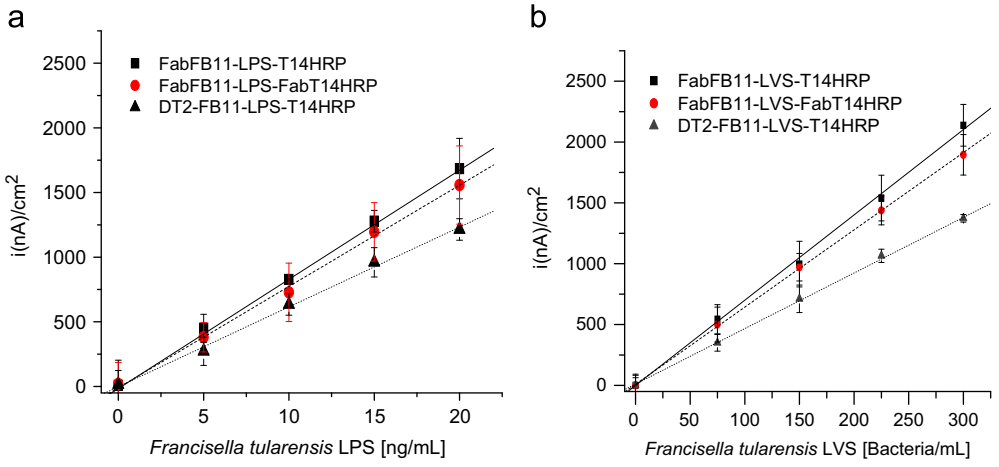
Both whole antibodies and antibody fragments modified with horseradish peroxidise (HRP) were used as secondary labelled antibodies in combination with immobilised F(ab) fragments by modification with maleimide activated horseradish peroxidise (HRP) (Hermanson, 2008). The conjugates were evaluated using ELISA.

The F(ab)FB11 fragment was used as a capture antibody and F(ab)T14 fragment modified with HRP and T14-HRP served as secondary HRP labelled antibody. The results obtained from the ELISA demonstrated that the produced fragments were still active after fragmentation and chemical reduction, and, in the case of the labelled antibody, after chemical linkage to HRP (Supplementary information Fig. S5). The optimum concentrations were elucidated, achieving limits of detection of 6.2 and 5.8 μg /mL, for the labelled F(ab)T14 and labelled whole T14 antibody, respectively.

### 3.3. Development of electrochemical immunosensor

For development and testing of the immunosensor, a conventional three-electrode cell was used, where 3 mm-diameter macrolithographically produced gold electrodes were used as working electrodes with a Ag/AgCl reference and Pt counter electrode. The electrochemical immunosensor was constructed using the FB11 whole antibody/F(ab) fragments as capture antibody and whole T14/F(ab) T14 fragments as secondary labelled antibodies. This sensor was used to detect different concentrations of the LPS antigen isolated from *F. tularensis* as well as for the detection of whole (inactivated) bacterial cells of *F. tularensis* LVS.

The immunosensor exploiting a F(ab)FB11 fragment SAM was more sensitive, with $>2$ times fold higher signal observed for the detection of whole bacterial cells. This can be attributed to a better orientation of the fragment on the surface directly exposing the antigen binding site, facilitating more efficient antigen binding, combined with the antibody enzyme label being closer to the electrode interface, thus giving a better response (Fig. 2b). The use of labelled antibody fragment as secondary antibody was not




| Immunosensor format | L.O.D *F. tularensis* | | | | Sensitivity | |
|---|---|---|---|---|---|---|
| | LPS (ng/mL) | RSD (%) | LVS (bacteria/mL) | RSD (%) | LPS (nA.mL/ng.cm$^2$) | LVS (nA.mL/bacteria.cm$^2$) |
| DT2- FB11 T14-HRP | 5.3 | 9.9 | 37 | 9.8 | 62 | 4 |
| F(ab)FB11 T14-HRP | 4.5 | 9.5 | 31 | 9.4 | 83 | 8 |
| F(ab)T14 F(ab)-HRP | 6.5 | 9.7 | 42 | 9.6 | 79 | 6 |

**Fig. 2.** Amperometric responses of the whole antibodies immobilised via chemical cross-linking to a bipodal alkyl thiol SAM and SAM formed from the direct chemisorption of antibody fragments F(ab) to different LPS and LVS of *F. tularensis* concentrations. Each data point represents the average of three independent measurements.

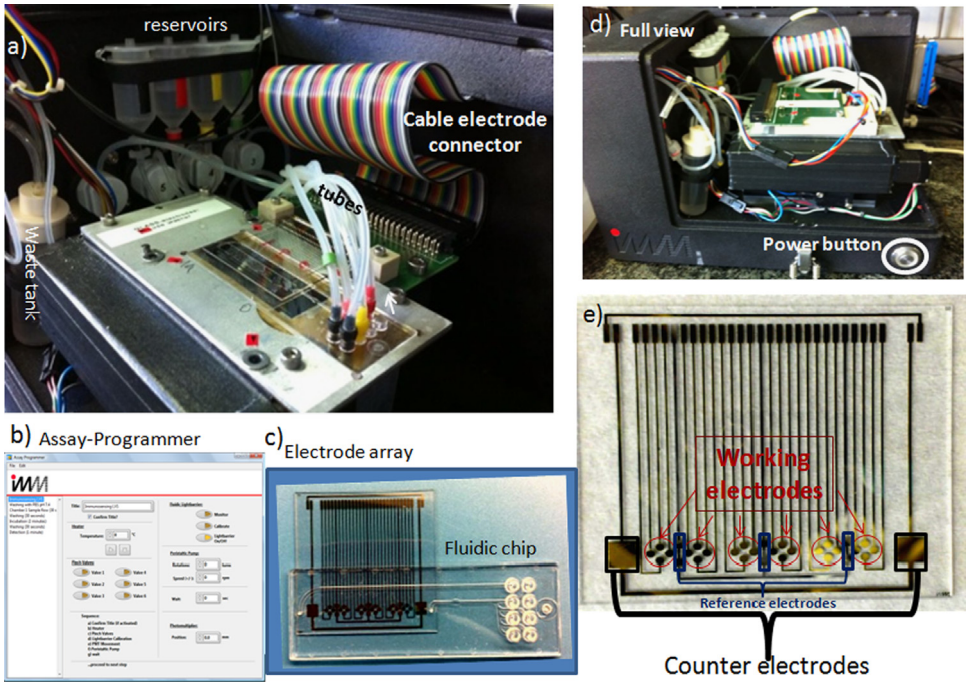



**Fig. 3.** The amperometric immunosensor detection set-up. The set-up contains a peristaltic pump positioned behind the reservoirs to flow the solutions into electrode array mounted within the microfluidics. (a) the electrode array with microfluidics placed in the platform and connected to the potentiostat for amperometric measurement; (b) a sample script-based assay program; (c) the electrode array integrated with microfluidics; (d) a full front view of the tester set-up device; and (e) lithographically produced gold electrode array with internal reference electrode and counter electrode.

observed to be significantly different to that of the use of the whole labelled antibody.

### 3.4. Fluidics and set-up

Following demonstration of the functionality of the developed immunosensor for the detection of both the LPS antigen as well as the inactivated bacterial cells, an electrode array was functionalised and housed within microfluidics (Fig. 3), within a tester set-up consisting of several reservoirs for analytes and buffers, silicone tubes, waste tanks, fluidics, and controlled by a script-based assay programme. The whole set-up enabled complete automation of the immunosensor as well as facilitating the use of smaller quantities of reagents. Electrode functionalisation with the optimum surface platform, i.e. F(ab)FB11 fragment was carried out outside the microfluidics set-up. Following the modification process, the electrode array was housed in the microfluidic set-up (Fig. 3c) using an adhesive gasket which acts as spacer between the polycarbonate microfluidic chip and the glass electrode. This generates a flow cell above the electrode area with a width of 4.5 mm, a height of 0.1 mm and a total length of 24 mm, housing 24 individual working electrodes (which could be separately functionalized, allowing multiplex detection and/or the incorporation of controls). The flow cell has an inlet resp. outlet with a diameter of 1 mm that is connected with a feeding channel with a width of 0.8 mm and a height of 0.3 mm leading to tube inlets resp. a waste outlet. The silicone tubes connected to the reservoirs were connected to the tube inlets of the microfluidic set-up via Mini-Luer adapters (supplied by microfluidic ChipShop GmbH), allowing flowing of different liquid samples as required throughout the assay. The four reservoirs available were used to store pre-mixed LVS and T14-HRP labelled antibody, PBS, TMB substrate, and Milli-Q water for washing. The flowing of liquids over the electrode was carried out automatically using the script based assay programme, which was also used to process the data obtained.

An optimisation of the incubation time outside and inside the microfluidic set-up was carried out. Interestingly, incubation of the target inside the fluidic cell resulted in an increased current response, even though smaller electrodes are used. This is due to the fact that incubation of antigen sample inside the fluidic cell is more favourable to the formation of the immunocomplex. This can be explained by an easy direction of the antigen towards the binding site of the immobilised capture antibody sensing probe which is confined to a very small channel and not the large volume used outside the microfluidics in the developmental work. Furthermore, the required incubation time was vastly reduced when the immunosensor was housed within the fluidics (Fig. 4b), and just 2 min incubation was required, as compared to a 10 min incubation outside the fluidics (Fig. 4a). The microfluidic set-up also allowed a tight control of temperature during the assay and to this end, the effect of temperature on sensor performance was evaluated and no great differences were observed at the temperatures studied (Fig. 4c).

The incubation time was also optimised for the detection of the *F. tularensis* bacterial cells, and similar results were obtained and again 2 min exposure was observed to be adequate. A 15 min incubation was required for the labelled secondary antibody (Fig. 4d), and together with the automated washing steps after each incubation as well as automated substrate addition and measurement, the duration for performance of the overall assay was 22 min (Fig. 5).

In order to further shorten the length of the assay, the target bacterial cells and the labelled secondary antibody were mixed together at different pre-incubation times before exposure to the immunosensor. A 15 min pre-incubation of the bacterial cells and HRP-labelled mAb gave equivalent response to that obtained when the antigen and labelled antibody were added sequentially with an intermittent washing step. The length of exposure of this pre-mixed solution on the electrochemical sensor was 2 min, resulting in a total assay time of ca.18 min, with only one required washing step, thus simplifying the fluidic requirements.

Finally, using the optimised incubation time and temperature, and pre-mixing the bacterial cells and the labelled reporter antibody, different concentrations of whole bacterial cells were detected using

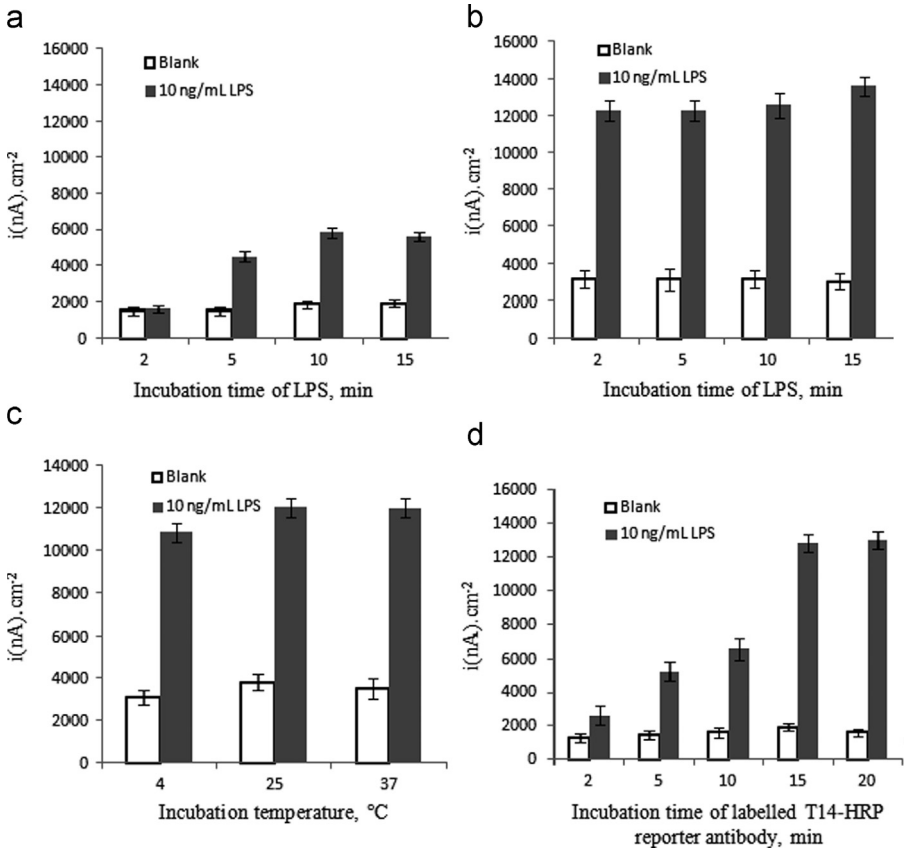



**Fig. 4.** Step and Sweep (SAS) amperometric response of the F(ab) fragment FB11 functionalised electrochemical immunosensor (a) to 10 ng/mL LPS sample outside the fluidics and (b) to 10 ng/mL LPS sample inside the fluidics at different incubation times of antigen-LPS, (c) to 10 ng/mL LPS sample inside the fluidics at different incubation temperatures and (d) different incubation times for the secondary labelled antibody T14HRP. Each data point represents the average of nine measurements on three separate electrode arrays.

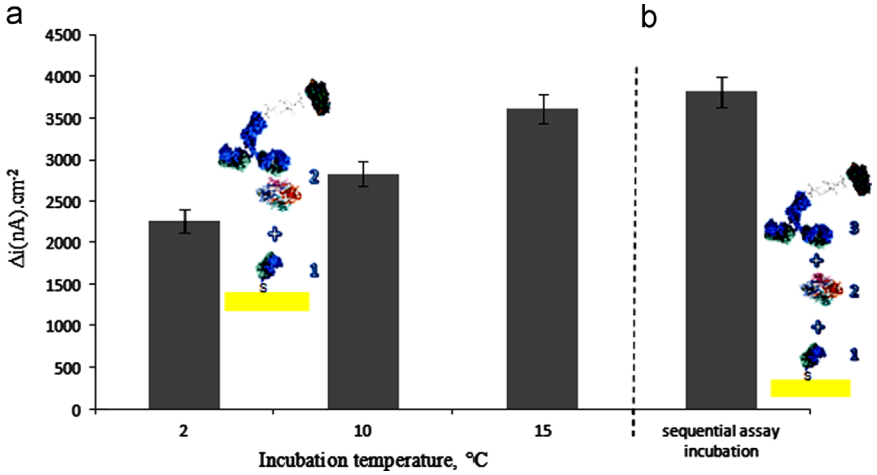



**Fig. 5.** Step and Sweep (SAS) amperometric responses of the direct F(ab) fragment FB11 immobilised based electrochemical immunosensor to a fixed concentration (150 bacteria/mL) of *F. tularensis* (LVS). (a) at different incubation times of pre-mixed LVS target and secondary labelled antibody T14HRP; (b) sequential addition of LVS (2 min) and T14-HRP (15 min) with intermittent washing step. Each data point represents the average of nine measurements on three separate microchip array.

the developed automated set-up with fragment F(ab)FB11 as capture antibody and whole mAb T14-HRP as secondary labelled antibody. The instrumentation used for detection was fully automated using script-based programming after insertion of the immunosensor array (1 mm Ø). The buffer solutions for washing and the sample mixed with T14-HRP were stored in each of the reservoirs and the solutions the array was exposed to the different solutions through microfluidics via the peristaltic pump. The whole *F. tularensis* LVS bacteria can be detected up to $1 \times 10^3$ bacteria/mL with a very low limit of detection of 38 bacteria/mL. This detection limit is far lower compared to previously reported immunosensors (Anderson et al., 2000; Kleo et al., 2012; Skladal et al., 2005) and even with reported ELISA results (Pohanka et al., 2008). In order to evaluate the sensitivity of the immunosensor using the whole set-up, the amperometric response was normalised taking into consideration the surface active area of the electrodes used, and a 3 fold increase in sensitivity as compared to the signal obtained outside the fluidics was observed (Fig. 2b). This highlights the important role microfluidics play in improving the

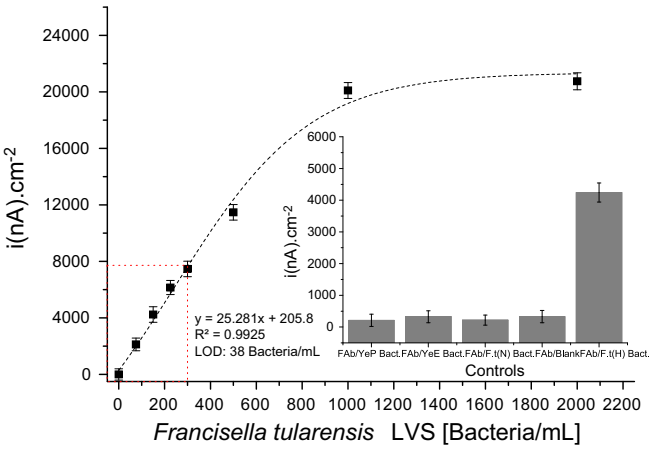



**Fig. 6.** Calibration curve and sensitivity of the electrochemical based-immunosensor detection system for the analysis of inactivated *F. tularensis* LVS subsp. *holarctica* bacteria. Step and Sweep (SAS) amperometric responses are shown for capture antibody on the chip-*F. tularensis* antibody FB11 F(ab) to different bacterial solutions which are flushed into the chip electrodes' surface via microfluidic device. Inset: Specificity of the immunosensor to *F. tularensis* LVS subsp. *holarctica* (*F.t* (H)Bact.), *F. tularensis* subsp. *novicida* (F.t (N)Bact.), *Yersinia enterocolitica* subsp. *enterocolitica* (YeE Bact.), *Yersinia pseudotuberculosis* (YeP Bact.). Each data point represents the average of three independent measurements.

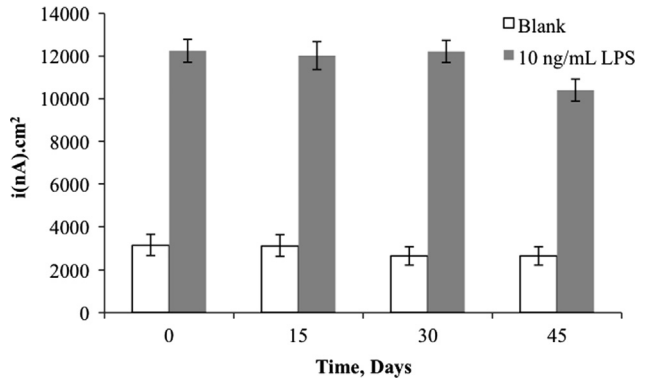



**Fig. 7.** Stability of stored Fab-modified sensors over a 45-day period. Sensors were stored at 4 °C and exposed to 10 ng/mL LPS every 15 days for 45 days. ($n$=3, RSD %=9.6).

sensitivity of the biosensor. The analysis time is approximately 18 min, comprising pre-incubation (15 min), automated addition of this pre-mixed solution to the housed electrode array and 2 min incubation, washing steps with buffer solution ($<30$ s) before automated substrate addition and electrochemical detection. The output signal was measured in less than 1 min. This short analysis time is advantageous in real situations such as bioterrorism events, and is far shorter than the analysis time required for ELISA (Vivekananda and Johnathan, 2006) or PCR (Sjostedt et al., 1997). Furthermore, the actual operator's hands-on time is less than a minute and simply requires rapid mixing of the sample containing the target with the labelled antibody in a reaction vessel e.g. Eppendorf tube and addition to the appropriate reservoir within the tester set-up.

To demonstrate the specificity of the immunosensor, control measurements were performed by exposing the modified electrode array with three different species (Fig. 6 inset). It was observed that F(ab)FB11 modified electrodes only obtained a specific sensor response when exposed to *F. tularensis* LVS subsp. *holarctica* bacteria. Other bacterial species, i.e. *Yersinia enterocolitica* subsp. *enterocolitica* and *Yersinia pseudotuberculosis*, including *F. tularensis* subsp. *novicida* did not give any response, highlighting the specificity of the developed immunosensor.

In order to show the true application of the immunosensor as an alarm sensor that would be integrated within a temperature controlled, automated microsystem linked, for example, to an automated air sampler, the stability of the immobilised F(ab) fragments on the gold electrode array was evaluated over a 45-day period. Electrode arrays were stored at 4 °C. As can be seen in Fig. 7, the electrodes modified with the Fab fragments showed a practically constant amperometric response to fixed concentration of 10 ng/mL of LPS target over 30 days, with only a slight loss of antigen recognition activity observed after 45 days, indicating the high stability of the immobilized Fab fragments. It can thus easily be envisaged that the sensor arrays could be replaced on a monthly basis from an automated sampling site.

## 4. Conclusions

This report details the development of an electrochemical immunosensor for the automated detection of *F. tularensis* LVS subsp. *holarctica* based on F(ab) fragments on Au surfaces as it gave better sensitivity compared to chemisorbed self-assembled monolayer cross-linked whole antibody. Antibody-enzyme conjugates were prepared in-house using whole antibody as well as antibody fragments, and similar results obtained in both cases. The immunosensor was used for the detection of the lipopolysaccharide antigen as well as *F. tularensis* LVS bacterial cells. The F(ab) fragments retained ~85% of antigen recognition ability after 45 days of storage at 4 °C. The developed electrochemical immunosensor was then transferred to an automated microfluidic set-up housed within a tester set-up and the assay parameters were optimised and the required incubation time within the fluidic set-up was reduced from 10 to 2 min for the target and 15 min for the labelled secondary antibody. Washing steps were reduced by mixing the bacterial cells sample with the labelled antibody and incubated in one of the reservoirs for 15 minutes before being introduced to the electrode array for signal read-out. The specificity of the immunosensor has been clearly determined and no cross-reactivity has been found.

This report is the first stage of development of a platform for the multiplexed detection of a range of bioterrorism agents (i.e., *Bacillus anthracis*, *Brucella abortis and melitensis*, *Bacteriophage lambda*, *Burkholderia mallei and pseudomallei*, *Coxiella burnetii*, *Yersinia pestis*, *and Bacillus thuringiensis*) and the electrode arrays used in this work has been designed to allow incorporation of multiple antibodies immobilised on individual electrodes for the simultaneous detection of the listed bioterrorism agents and work is ongoing to achieve this goal. The applicability of the developed system to real situations such as an early detection of bioterrorism events is very promising as the obtained LOD was very low and the assay time quite short. This facilitates a rapid alarm based detection of *F. tularensis* when linked e.g. to an air-sampling system. Ongoing and future work will focus on attempting to further reduce the sampling time, to increase the stability of the Fab functionalised sensors using stabilising agents and to apply to the analysis of real samples within specialised regulatory laboratories.

## Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme "The lab-free CBRN detection device for the identification of biological pathogens on nucleic acid and immunological level as lab-on-a-chip system applying multisensor technologies", MultisenseChip [FP7/2007-2013]. The authors thank microfluidic ChipShop (http://www.microfluidic-chipshop.com/) for provision of the microfluidics.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.03.024.

## References


Anderson, G.P., King, K.D., Gaffney, K.L., Johnson, L.H., 2000. Biosens. Bioelectron. 14 (10–11), 771–777.

Bystrom, M., Bocher, S., Magnusson, A., Prag, J., Johansson, A., 2005. J. Clin. Microbiol. 43 (10), 5355–5358.

Dennis, D.T., Inglesby, T.V., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, E., Parker, G., Perl, T.M., Russel, P.K., Tonat, K., 2001. J. Am. Med. Assoc. 285 (21), 2763–2773.

Emanuel, P.A., Bell, R., Dang, J.L., McClanahan, R., David, J.C., Burgess, R.J., Thompson, J., Collins, L., Hadfield, T., 2003. J. Clin. Microbiol. 41 (2), 689–693.

Fragoso, A., Latta, D., Laboria, N., von Germar, F., Hansen-Hagge, T.E, Kemmner, W, Gärtner, C., Klemm, R., Dreseb, K.S., O'Sullivan, C.K, 2011. Lab Chip 11, 625–631.

Grunow, R., Splettstoesser, W., McDonnald, S., Otterbein, C., O'Brien, T., Morgan, C., Aldrich, J., Hofer, E., Finke, EJ., Meyer, H., 2000. Clin. Diagn. Lab. Immunol. 7 (1), 86–90.

Hermanson, G.T., 2008. Bioconjugate Techniques, 2nd ed. Academic press, Oxford, UK.

Johansson, A., Ibrahim, A., Goransson, I., Eriksson, U., Gurycova, D., Clarridge, J.E, Sjostedt, A., 2000. J. Clin. Microbiol. 38 (11), 4180–4185.

Kleo, K., Schafer, D., Klar, S., Jacob, D., Grunow, R., Lisdat, F., 2012. Analy. Bioanal. Chem. 404, 843–851.

Lazcka, O., Javier Del Campo, F., Xavier Munoz, F., 2007. Biosens. Bioelectron. 22, 1205–1217.

Magnarelli, L., Levy, S., Koski, R., 2007. Res. Vetenerary Sci. 82, 22–26.

Nassef, H.M., Civit, L., Fragoso, A., O'Sullivan, C.K., 2009. Anal. Chem. 81, 5299–5307.

Penn, R.L., 2005. Mandell Douglas Bennett's Princ. Pract. Infect. Dis. 2, 2927–2937.

Pividori, M.I., Lermo, A., Bonanni, A., Alegret, S., del Valle, M., 2009. Anal. Biochem. 388, 229–234.

Pohanka, M., Skládal, P., 2008. J. Appl. Biomed. 6, 57–64.

Pohanka, M., Pavlis, O., Kroca, M., 2008. Def. Sci. J. 58 (5), 698–702.

Pohanka, M., Skládal, P., Kroèa, M., 2007. Def. Sci. J. 57 (3), 185–193.

Porsch-Ozcürümez, M., Kischel, N., Priebe, H., Splettsto sser, W., Finke, EJ., Grunow, R., 2004. Clin. Diagn. Lab. Immunol. 11 (6), 1008–1015.

Riddles, P.W., 1983. Methods Enzymol. 91, 49–60.

Savitt, A.G., Mena-Taboada, P., Monsalve, G., Benach, J.L., 2009. Clin. Vaccine Immunol. 16 (3), 414–422.

Simşek, H., Taner, M., Karadenizli, A., Ertek, M., Vahaboğlu, H., 2012. Eur. J. Clin. Microbiol. Infect. Dis. 31 (9), 2353–2357.

Sjostedt, A., Eriksson, U., Berglund, L., Tarnvik, A., 1997. J. Clin. Microbiol. 35 (5), 1045–1048.

Skládal, P., Pohanka, M., Kupská, E., Šafář, B., 2010. Biosensors. INTECH, Vienna, pp. 115–126.

Skladal, P., Symerska, Y., Pohanka, M., Safar, B., Macela, A., 2005. Defense against Bioterror: Detection Technologies, Implementation Strategies and Commercial Opportunities, pp. 221–232.

Su, J., Yang, J., Zhao, D., Kawula, T.H., Banas, J.A., Zhang, J.R., 2007. Infect. Immun. 75 (6), 3089–3101.

Versage, J.L., Severin, D., Chu, M.C., Petersen, J.M., 2003. J. Clin. Microbiol. 41, 5492–5499.

Vivekananda, Jeevalatha, Johnathan, L., 2006. Lab. Investig. 86, 610–618.

Warsinke, A., Benkert, A., Scheller, F.W., 2000. J. Anal. Chem. 366 (6-7), 622–634.