



Ultrasensitive peptide-based multiplexed electrochemical biosensor for the simultaneous detection of *Listeria monocytogenes* and *Staphylococcus aureus*

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Abstract

A novel electrochemical biosensor is reported for simultaneous detection of two of the most common food-borne pathogens: *Listeria monocytogenes* and *Staphylococcus aureus*. The biosensor is composed of an array of gold nanoparticles-modified screen-printed carbon electrodes on which magnetic nanoparticles coupled to specific peptides were immobilized via streptavidin-biotin interaction. Taking advantage of the proteolytic activities of the protease enzymes produced from the two bacteria on the specific peptides, the detection was achieved in 1 min. The detection was realized by measuring the percentage increase of the square wave voltammetric peak current at 0.1 V versus a Ag/AgCl reference electrode in ferro/ferricyanide redox couple after incubation with the bacteria protease. The integration of the specificity of the bacterial enzymes towards their peptide substrates with the sensitivity of the electrochemical detection on the sensor array allows the rapid, sensitive and selective quantification of the two bacteria. Outstanding sensitivities were achieved using this biosensor array platform with limit of detection of 9 CFU mL⁻¹ for *Listeria monocytogenes* and 3 CFU mL⁻¹ for *Staphylococcus aureus*. The multiplexing capability and selectivity of the array voltammetric biosensor were demonstrated by analysing samples of *Staphylococcus aureus*, *Listeria monocytogenes* or *E. coli* and also containing a mixture of two or three bacteria. Using this biosensor, the two bacteria were successfully quantified simultaneously in one step without the need for DNA extraction or amplification techniques. This platform offers promise for rapid, simple and cost-effective simultaneous detection of various bacteria.

Keywords Multiplexed detection · Square wave voltammetry · Magnetic nanobeads · Biotinylated peptide · Array electrodes · Gold nanoparticles

Introduction

Food-borne bacterial pathogens are the main sources of many food poisoning cases usually due to improper food handling causing serious illness and sometimes can lead to death [1]. The Center for Disease Control and Prevention (CDC) had identified eight known pathogens that account for the majority

of food-borne illness. Among them, *Staphylococcus aureus* (*S. aureus*) [2] and *Listeria monocytogenes* (*L. monocytogenes*) [3] stand out as common causes of food-borne infections.

S. aureus is a gram-positive bacterium commonly found on the skin of healthy people and animals [4]. However, when it is transmitted to food products such as dairy products and meat, it can multiply and produce harmful toxins which are heat resistant and cannot be destroyed by cooking [5]. Infection by *S. aureus* causes symptoms including stomach cramps, nausea, vomiting or diarrhoea [6]. It causes serious health issues particularly for people with low immunity and those with chronic conditions such as cancer, diabetes, vascular disease and lung disease. *L. monocytogenes* is an intracellular gram-positive bacterium which has been widely implicated within the past decade as the cause of several outbreaks [7]. Eating food contaminated with *Listeria* causes listeriosis [8], a serious infection with high mortality rate mainly among

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older adults, pregnant women, their fetuses and immunocompromised persons [9]. Infection with *Listeria* causes symptoms of abortion, neonatal death, meningitis and septicaemia [10]. *Listeria* can be found in water, soil, ready-to-eat food, unpasteurized milk, raw meat and vegetable, and it can grow in refrigerators unlike most other bacteria [11].

The detection of pathogenic bacteria such as *L. monocytogenes* and *S. aureus* was usually achieved using the conventional cultural methods [12]. However, this method is time-consuming as it takes few days to obtain the results. Immunoassays such as enzyme-linked immunosorbent assays (ELISA) have been also used for the detection of various bacteria [13]. Immunoassays require less time to perform, but they usually suffer from the poor sensitivity compared with the cultural method. Amplification techniques such as polymerase chain reaction (PCR) have been alternatively used [13]. However, PCR requires expensive reagents, sample enrichment and extraction steps prior to amplification, and thus, it must be performed in specialized laboratories. Thus, simple, fast and low-cost methods that are suitable for on-site detection of bacteria are highly demanded for food safety and infection control particularly in developing countries and remote areas.

Biosensors represent extremely valuable tools for portable, fast and sensitive detection of bacteria for on-site monitoring [14]. Therefore, several types of biosensors have been developed for the detection of *S. aureus* [15] and *Listeria* [16] based on colourimetric [17–20], surface plasmon resonance [17–22], surface-enhanced Raman spectroscopic [23, 24], fluorescence [25–32], impedimetric [33–39] and amperometric [40–45] techniques. However, despite the tremendous progress offered by these biosensors for bacteria detection, they still need to overcome major limitations to be widely utilized for field applications. For the optical biosensors, the major challenges include the high cost of the equipments, the general requirement for biomaterial labelling and difficulty of miniaturization. Electrochemical biosensors, in particular, offer low-cost, high sensitivity, label-free detection and great potential for miniaturization and integration in portable devices for on-site detection.

However, the electrochemical biosensors developed for *S. aureus* or *Listeria* detection are mainly based on antibodies [21, 33, 34] or DNA [40, 45]. The production of antibodies is time-consuming and costly and suffers from batch-to-batch variations. On the other hand, the DNA as recognition receptor is cheap and can be easily synthesized, but the detection requires extraction step for the bacterial DNA which makes it hard to be integrated in portable devices.

Recently, virulence proteases produced from bacteria were used as biomarkers for the presence of some bacteria [46, 47]. Each protease enzyme has the capability to break a peptide bond at specific site, and thus, synthetic peptide sequences can be used as specific recognition receptors for each bacterium

[48]. This principle has been exploited for the semi-quantitative colourimetric detection of different bacteria [46, 47]. However, in order to achieve accurate quantification, multiplexed electrochemical biosensor of bacteria would provide more information in less time. To the best of our knowledge, there is no electrochemical biosensor capable of performing multiplexed detection of bacteria using peptide as recognition receptor and protease enzyme as marker that has been developed so far.

In this article, we present a novel electrochemical biosensor for multiplexed detection of both *L. monocytogenes* and *S. aureus*. By selectively functionalizing the array electrodes with specific peptides for both bacteria, we were able to identify each bacterial protease. The cleavage of the peptide by the protease causes a dissociation of the magnetic nanoparticles from the electrode surface which can be monitored by following the change in the SWV reduction peak current. This biosensing principle enables not only very fast and accurate detection but also the independent interrogation of each electrode simultaneously which facilitates the multiplexed analysis of different bacteria in a simple manner. Therefore, this biosensing platform enables a rapid, simple and efficient detection of bacteria.

Experimental section

Materials and reagents

The *S. aureus* biotinylated peptide sequence (NH₂-Ahx-ETKVEENAIQK Ahx-biotin) and the *Listeria* peptide sequence (NH₂-Ahx-NMLSEVERE-Ahx-biotin) were custom synthesized by Pepmic Co., Ltd. (Suzhou, China). Carboxylate-modified magnetic nanoparticles (around 50 nm in diameter) were obtained from Turbobeads (Zurich, Switzerland). 11-Mercaptoundecanoic acid (MUA), bovine serum albumin (BSA), streptavidin, ethanol amine, N-hydroxysuccinimide (NHS), potassium ferrocyanide (K₄Fe(CN)₆), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), potassium ferricyanide (K₃Fe(CN)₆), dipotassium hydrogen orthophosphate, sodium chloride, potassium dihydrogen orthophosphate, *Listeria monocytogenes* (ATCC 19115), *S. aureus* (ATCC 25923) and *E. coli* O157:H7 were purchased from Sigma Aldrich (Ontario, Canada). Sterile filters with pore size of 0.22 µm were purchased from EMD Millipore (Alberta, Canada). Brain heart infusion (BHI) agar and broth were obtained from SDA Oxoid, Ltd. (Basingstoke, UK). To prepare 10 mM of the 1:1 redox couple solution, phosphate buffer saline (PBS), pH 7.4 was used. The solutions of the peptides were prepared in DMSO and diluted using PBS buffer pH 7.4. The activation step of the carboxylated magnetic nanobeads was performed with EDC/NHS solution in PBS buffer pH 5.5. The electrodes

were blocked with 1% BSA in PBS buffer pH 7.4. Aqueous solution of ethanol amine pH 8.0 was used to block the free carboxylic moieties on the magnetic particles. All the aqueous solutions were prepared in Milli-Q grade water.

Instrumentation

The electrochemical measurements (cyclic and square wave voltammetry) were carried out using an Autolab potentiostat, PGSTAT302N (Eco Chemie, Netherlands). The potentiostat was connected to a personal computer and operated by 1.11 NOVA software. The sensors were fabricated on disposable electrical printed (DEP) microarray electrodes obtained from BioDevice Technology (Nomi, Japan). The array electrode composed of a 3-electrode configuration with eight individually addressable carbon working electrodes (round shape), a ring-shaped carbon counter electrode and a central silver/silver chloride reference electrode. The sensor connector employed to connect the DEP electrodes to the potentiostat was obtained from BioDevice Technology.

Methods

Extraction of protease enzymes from the *S. aureus*, *L. monocytogenes* and *E. coli* bacteria cultures

Listeria monocytogenes, *S. aureus* and *E. coli* bacteria strains were streaked individually on BHI agar plates and incubated at 37.0 °C for 24 h. After that, a single colony from each plate was picked up and inoculated into BHI broth overnight at 37 °C. The bacterial colony-forming unit (CFU mL⁻¹) values of the three bacterial cultures were calculated using viable count spread dilution method after serial dilution of the primary bacterial culture (PBC). The protease enzyme solutions were obtained from the serial dilutions of each bacterial culture by centrifugation at 3000×g for 10 min. After the centrifugation, the bacteria cells were separated as pellets, and the remaining supernatants were filtered through syringe filters (22 µm pore size). By performing Universal Protease Activity Assay using casein as substrate, a good correlation between the protease proteolytic activity and the concentration of each bacterium was observed.

Attachment of *S. aureus* and *Listeria* peptides on the magnetic nanobeads

The attachment of the peptides to the magnetic nanoparticles were performed using the protocol reported previously [47]. The carboxylated magnetic nanoparticles were activated using EDC/NHS chemistry and then incubated with the peptide-modified nanoparticles.

Functionalization of the carbon array electrodes and fabrication of the *S. aureus* and *Listeria* multiplexed biosensor

The fabrication of the multiplexed sensor is shown in schematic diagram (Fig. 1). First, gold nanoparticles are electrodeposited on the eight carbon electrodes of the array chip by covering the chip surface with a solution of 150 µL of 6 mM HAuCL₄ in 0.1 M KNO₃ and applying twenty CV scans from -0.2 to -1.2 V at 50 mV s⁻¹ until a golden surface is observed. The AuNPs-modified electrodes were characterized using scanning electron microscope as shown in Fig. S1. The electrodes were then incubated in a solution of 1 mM of MUA in ethanol overnight at room temperature in a water-saturated atmosphere. The modified electrodes were washed with ethanol and then activated by incubation in 40 mg of EDC and 15 mg of NHS solution in PBS buffer pH 5.5 for 1 h. The electrodes were then washed and incubated in 10 µg mL⁻¹ streptavidin solution in PBS buffer pH 7.4 for 3 h at room temperature. The excess streptavidin was then washed with PBS buffer, and the surface was blocked with 1% BSA in PBS buffer pH 7.4.

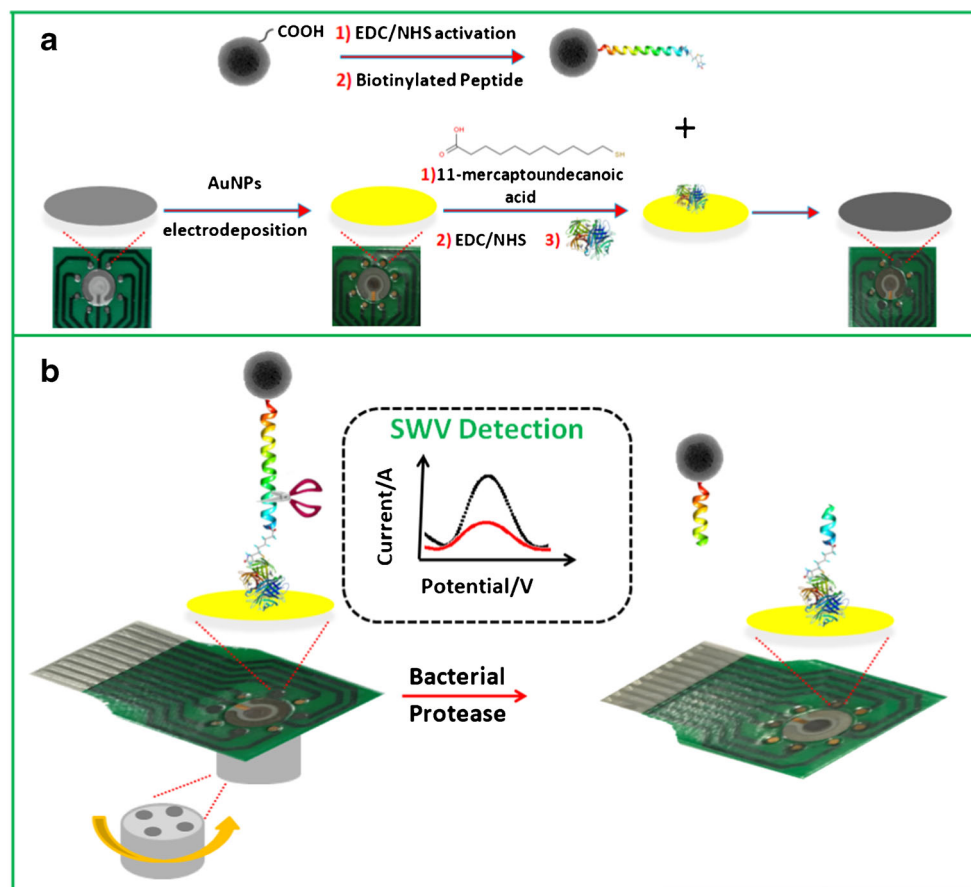
After the preparation of the streptavidin-modified electrodes, the two peptide-magnetic nanoparticle conjugates were attached on different electrodes on the same chip by placing the conjugate solutions over each individual electrode and incubation for 1 h at 37 °C. As shown in Fig. 1a, only 4 electrodes from the 8 working electrodes were coated with the peptide/magnetic particle conjugates (2 electrodes were coated with the *S. aureus* peptide/magnetic particles solution, and the other two electrodes were coated with *Listeria* peptide/magnetic particles to perform duplicate measurements). The 4 peptide/magnetic bead-coated electrodes show black colour unlike the golden yellow coloured uncoated electrodes (Fig. 1a). The excess magnetic particles were then removed by washing with PBS buffer or by placing magnetic over the electrode surface. Now the sensor chip is ready to use and can be stored dry at 4 °C until further use.

Cyclic voltammetry (CV) at a scan rate of 100 mV s⁻¹ within a potential range of -0.6 to 0.5 V and square wave voltammetry (SWV) within a range of 0.3 to -0.5 V were used for the characterization of the biosensor fabrication steps in 5 mM ferro/ferricyanide redox solution.

Biosensing experiments for *S. aureus* and *Listeria*

The detection experiments were carried out by placing 2 µl of each protease enzyme solution from *S. aureus* or *Listeria* bacterial cultures (from 10¹ to 10⁸ CFU mL⁻¹) on its specific electrode on the chip with incubation for 1 min at room temperature. Then, the cleaved peptide/magnetic nanoparticles were removed from the array electrode surface by washing with PBS buffer pH 7.4 or by rotating a magnet holder

Fig. 1 Schematic diagrams of the multiplexed array biosensor. **(a)** The preparation of the peptide/magnetic particles and the fabrication steps of the array biosensor. **(b)** The square wave voltammetry detection of the bacterial proteases through the cleavage of their specific peptides from the sensor surface and the release of the magnetic nanoparticles



underneath the chip. The magnetic holder is designed by inserting 4 magnet rods, aligned under the 4 working electrodes on the array chip, in a cylindrical Teflon holder. By rotating the magnet holder, the cleaved magnetic particles will move from the functionalized black electrode to the unfunctionalized golden electrode. Then, the 4 functionalized electrodes (2 for each analyte) were subjected to SWV measurements in 5 mM ferro/ferricyanide redox solution in PBS buffer. The SWV measurements were recorded at an interval time = 0.04 s, frequency = 25 Hz, a scan rate of 125 mV s⁻¹, amplitude = 20 mV and step potential = -5 mV. The biosensor response was determined by following the change in the SWV reduction peak current at 0.1 V versus Ag reference electrode before and after each peptide cleavage $(i - i^0)/i^0\%$, where i^0 is the peak current of either the *S. aureus* or *Listeria* biosensor before incubation with its specific protease and i is the peak current after incubation with each protease solution from different bacteria concentrations.

Optimization of the incubation period of the *S. aureus* and *Listeria* protease solutions on the biosensor

The incubation period of the *S. aureus* and *Listeria* proteases was optimized by incubating 2 µl of each protease

solution on its corresponding electrode for different periods ranging from 0.5 to 10 min. After each incubation period, the magnets were rotated underneath the chip as explained above to pull the cleaved peptide/magnetic particles away from the surface. Then, 150 µl of the redox couple solution was added on the chip surface, and SWV was recorded as explained above.

Selectivity experiments for the *S. aureus* and *Listeria* multiplexed biosensor

Two microlitre of each protease solution (from 10³ CFU mL⁻¹ of either *S. aureus*, *Listeria* or *E. coli*) were placed on the *S. aureus* and *Listeria* electrodes for 1 min at room temperature. The *S. aureus* and *Listeria* electrodes were also incubated with a mixture of the three proteases or two of them as well as 1% BSA protein as control. Then, the cleaved peptide-magnetic nanoparticles were pulled away from the electrode surface by rotating the magnet holder, and SWV scans were recorded in each case. The biosensor responses to the various bacteria types, mixtures and BSA were compared by determining the percentage change in the reduction peak current after cleavage.

Results and discussion

The working principle of the multiplexed array electrochemical biosensor

The *S. aureus* and *Listeria* multiplexed biosensor was developed on an array of screen-printed carbon electrodes as shown in Fig. 1. Gold nanoparticles were electrodeposited on the eight carbon working electrodes following the protocol reported and optimized previously [49] in order to realize larger surface area and higher conductivity. The deposition of the gold layer was visually observed as shown in Fig. 1a. Then, self-assembly monolayer of MUA was deposited on the AuNPs-modified electrodes and activated with EDC/NHS chemistry to enable the covalent immobilization of streptavidin protein on the surface.

Protease enzymes were used here as biomarkers for each bacteria. Each protease causes cleavage in a specific peptide sequence which represents the basis of the detection principle. Two previously reported [47, 48] peptide substrates were used in this study for *S. aureus* and *Listeria*. The peptide sequences were elongated from both sides using Ahx linker to minimize the steric hindrance and, thus, to enable the access of each protease to the cleavage site. The N-terminal of each peptide was activated with EDC/NHS to allow its covalent attachment to the carboxylate moieties on the magnetic nanoparticles. However, the other terminals of the peptides were modified with biotin to enable the attachment of the peptide/magnetic particle conjugate to the streptavidin-modified electrode surface. Here, we attached the *S. aureus* and *Listeria* peptide/magnetic bead conjugates only on 4 electrodes from the chip to enable the simultaneous detection of the two bacteria strains. The colour contrast between the unfunctionalized

golden yellow electrodes and the functionalized black electrodes can be clearly seen (Fig. 1a). The incubation of *S. aureus* and *Listeria* protease solutions on their specific electrodes causes a cleavage of the relevant peptide in 1 min. By rotating the magnets placed underneath the functionalized electrodes, the cleaved peptide/magnetic beads were pulled away from the electrode surfaces which lead to an increase in the SWV reduction peak current of the redox couple (Fig. 1b) which was employed for the detection.

Voltammetric characterization of the preparation steps of the multiplexed peptide/magnetic nanoparticle sensor

Cyclic voltammetry (Fig. 2a) and SWV (Fig. 2b) techniques were used to confirm the stepwise modification of the array electrodes and the successful fabrication of the biosensor. As shown in Fig. 2a, the CV of the bare carbon electrode showed quasi-reversible peaks in the ferro/ferricyanide redox solution. The AuNPs-modified electrodes exhibit a dramatic enhancement in the CV anodic and cathodic peak currents and a significant decrease in the peak-to-peak separation indicating the increase in the electron transfer rate and the electrode conductivity. After the incubation with MUA, a significant decrease in the CV peak currents was observed indicating the successful self-assembly of the MUA on the gold surface via its thiol groups. The negatively charged carboxylate groups of the MUA cause an electrostatic repulsion to the redox anion leading to the hindrance of the access of the redox molecules to the electrode surface and, thus, a decrease in the electron transfer rate. On the other hand, as shown in the inset of Fig. 2a, the reaction with the streptavidin molecules after the activation of the carboxylate groups caused an increase in the peak current.

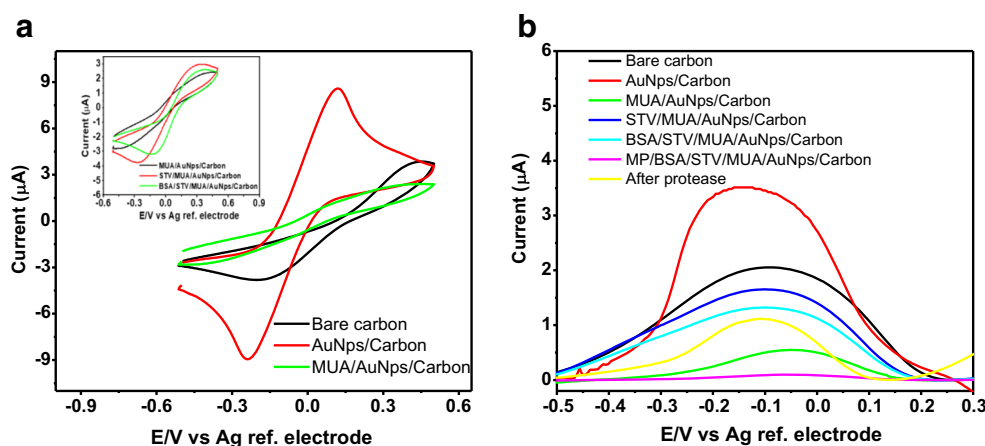


Fig. 2 Characterization of the fabrication steps of the multiplexed biosensor using cyclic voltammetry (a) and square wave voltammetry (b). The curves represent the bare carbon electrode, the gold nanoparticles-modified electrode, after the self-assembly of mercaptoundecanoic acid, after streptavidin immobilization, after BSA blocking, after the peptide/magnetic particle conjugate binding and after

the addition of protease solution from 10^8 CFU mL^{-1} *S. aureus*. The measurements were carried out in 5 mM $[\text{Fe}(\text{CN})_6]^{4-/3-}$ redox solution in PBS buffer pH 7.4. The CV scan rate was 100 mV s^{-1} . The SWV was performed using amplitude of 0.1 mV, interval time: 0.04 s, frequency: 25 Hz, a scan rate of 125 mV s^{-1} and a step potential of 0.01 V

Fig. 3 Representative SWV signals of the *S. aureus* (a) and the *Listeria* (b) sensors before and after 1-min incubation with the respective protease solution. Effect of incubation time of the *S. aureus* (c) or *Listeria* (d) protease enzyme from 10^3 CFU mL⁻¹ of bacterial culture solutions on the biosensor response measured as the percentage change in the SWV reduction peak current ($(i - i^0)/i^0\%$, where i is the peak current after protease incubation and i^0 is the peak current of the biosensor. The error bars represent the standard deviations of triplicate measurements

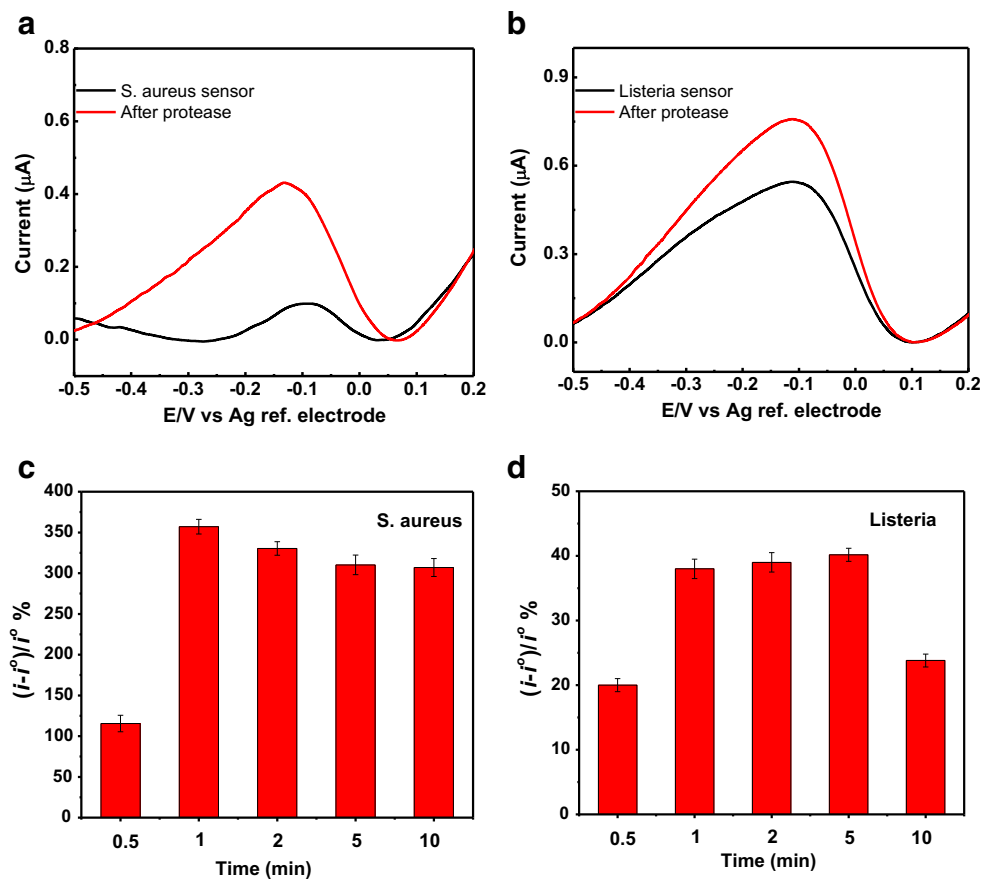
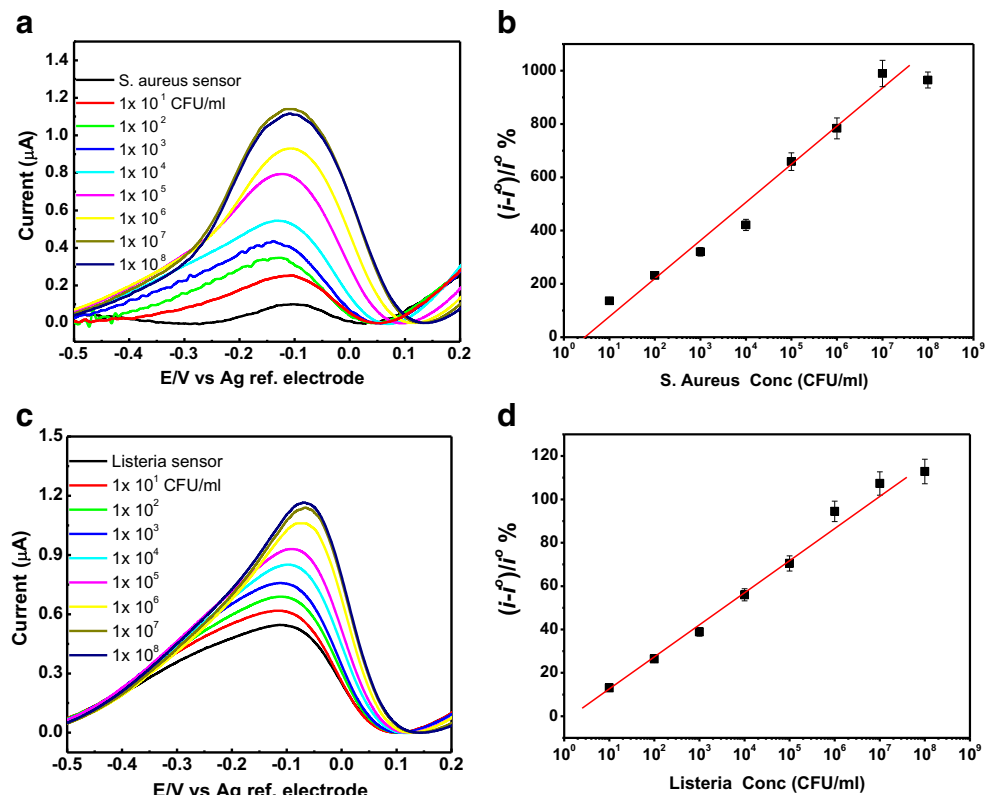


Fig. 4 The electrochemical response of the array biosensor towards *S. aureus* (a, b) and *Listeria* (c, d) proteases. The change in the SWV reduction peaks upon incubation of the array biosensor with protease enzyme solution from serial dilutions of *S. aureus* (a) or *Listeria* (c) bacterial cultures from 10 to 1×10^8 CFU mL⁻¹. The calibration plots for *S. aureus* (b) and *Listeria* (d) detection (a plot of $(i - i^0)/i^0\%$ versus the concentration of either *S. aureus* or *Listeria*). The error bars represent the standard deviations of triplicate measurements



This is likely attributed to the neutralization of some of the carboxylate groups on the surface with the streptavidin protein which shows a neutral charge at pH 7.4 [50]. Then, the blocking of the surface caused a decrease in the peak current again because of the bulky size of the BSA protein.

Figure 2b shows the SWV signals of the fabrication steps. As shown in the figure, the SWV signals were in agreement with the CV signals. The AuNPs-modified electrodes showed an increase in the reduction peak current compared with the bare carbon electrode. The modification with MUA caused significant decrease in the peak current which was enhanced after the immobilization of the streptavidin. Then, blocking with the BSA caused a decrease in the current. The binding of the biotinylated peptide/magnetic nanoparticle conjugate on the electrodes caused a further decrease in the peak current likely due to the carbon coating of the metallic nanoparticles which results in a decrease of the electron transfer rate. However, the addition of the specific protease enzyme solution to the biosensor caused a significant enhancement of the peak current. This increase in the peak current is attributed to the

cleavage of the peptide and the dissociation of the magnetic particles from the electrode surface leading to more access of the redox molecules to the gold electrode surface and enhancement of the peak current.

Optimization of the incubation period of the protease enzyme on the array electrochemical biosensor

It was important to optimize the detection time as it is a crucial parameter that highly affects the practical application of a biosensor. The electrodes of the array biosensor chip were incubated individually with protease enzymes from 10^3 CFU mL⁻¹ of *S. aureus* and *Listeria* for different periods ranging from 0.5 to 10 min. Then, the cleaved magnetic nanoparticles were removed from the surface by rotating the magnets. The array biosensor responses were calculated by determining the change in the SWV peak current before and after incubation with the proteases solutions. Figure 3 a and b show representative SWV signals of the *S. aureus* and the *Listeria* sensors before and after 1-min incubation with the respective protease solution. Figure 3 c and d show the *S. aureus* and the

Table 1 An overview on recently reported electrochemical methods for the determination of *Listeria monocytogenes* and *Staphylococcus aureus*

Bacteria strain	Recognition receptor	Detection technique	Electrode and nanomaterials used	Limit of detection	Reference
<i>S. aureus</i>	Antibody	EIS	Gold	10 CFU mL ⁻¹	[33]
<i>S. aureus</i>	Antibody	EIS	Gold	10 CFU mL ⁻¹	[34]
<i>S. aureus</i>	Aptamer	EIS	Reduced graphene oxide and gold nanoparticles	10 CFU mL ⁻¹	[35]
<i>S. aureus</i>	Peptides	EIS	Gold	10 ² CFU mL ⁻¹	[36]
<i>S. aureus</i>	—	EIS	Screen-printed electrodes	—	[37]
<i>S. aureus</i>	DNA	CV	Gold	10 fM synthetic DNA	[40]
<i>S. aureus</i>	Antibody	Amperometry	Gold	3.7×10^2 CFU mL ⁻¹	[41]
<i>S. aureus</i>	Antibody	Amperometry	Gold	2.3×10^3 CFU mL ⁻¹	[42]
<i>S. aureus</i>	Antibody	Amperometry	Gold	1.6×10^5 CFU mL ⁻¹	[43]
<i>S. aureus</i>	Peptide	SWV	Gold nanoparticle electrode	3 CFU mL ⁻¹	This work
<i>Listeria</i>	Immunosensor	EIS	Interdigitated microelectrode, magnetic and gold nanoparticles	1.6×10^2 CFU mL ⁻¹	[38]
<i>Listeria</i>	DNA	EIS	Platinum-modified glassy carbon electrode	—	[39]
<i>Listeria</i>	Antibody	EIS	Gold screen-printed electrode	—	
<i>Listeria</i>	DNA	Differential pulse voltammetry	Dendritic nanogold and reduced graphene-modified carbon ionic liquid electrode	2.9×10^{-13} mol/L	[45]
<i>Listeria</i>	Peptide	SWV	Gold nanoparticle electrode	9 CFU mL ⁻¹	This work

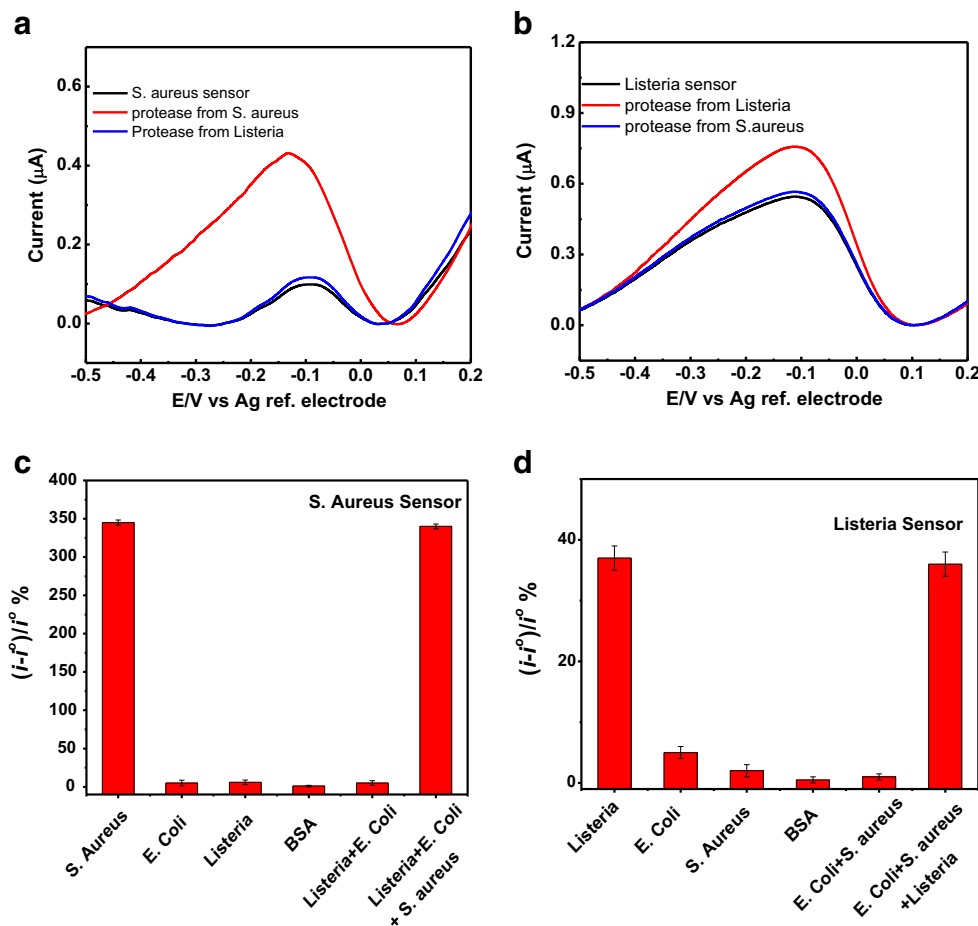
Listeria biosensor responses versus the detection time, respectively. In both cases, an increase in the biosensor response was observed until 1 min which was chosen for the detection experiments for both *S. aureus* and *Listeria*. However, longer incubation periods lead to a decrease in the biosensor response likely due to the non-specific adsorption of the protease enzymes on the electrode surface. This adsorption can shield the surface and hinders the access of the redox anions leading to less biosensor response.

Dose-response behaviour of the array biosensor for *S. aureus* and *Listeria* detection

Different concentrations of *S. aureus* and *Listeria* bacterial proteases ranging from 10 to 10^8 CFU mL⁻¹ were applied on the array chip in order to investigate the analytical range of the biosensor. At high concentration of bacteria, the cleavage of the magnetic particles causes an obvious change in the electrode colour from black to golden yellow which can be seen by the naked eye (Fig. 1b). Figure 4 a and c show the SWV signals of the *S. aureus* and *Listeria* biosensors before and after incubation with the specific proteases from different bacterial concentrations (10 – 10^8 CFU mL⁻¹) on their corresponding electrodes. As shown in the figure, a gradual

increase in the peak current was observed with increasing the concentrations of the bacteria in both cases. This is attributed to the cleavage of the peptide by the protease enzyme and the release of the magnetic nanoparticles from the surface causing enhancement of the electron transfer as explained above. The calibration plots (the biosensor response $((i - i^0)/i^0\%)$ versus the number of CFU mL⁻¹ of bacteria) for the *S. aureus* and *Listeria* detection on the array biosensor are shown in Fig. 4 b and d, respectively. As shown in the figure, linear responses were obtained from 10 to 10^7 CFU mL⁻¹ for both bacteria. The linear regression equations are: $(i - i^0)/i^0\% = 7.7 + 118.2 \log C$ [CFU mL⁻¹], $R = 0.98$ and $(i - i^0)/i^0\% = -2.2 + 14.8 \log C$ [CFU mL⁻¹], $R = 0.99$ for *S. aureus* and *Listeria*, respectively. The array multiplexed biosensor showed a very low limit of detection (LOD) of 3 and 9 CFU mL⁻¹ for *S. aureus* and *Listeria*, respectively. The LOD was calculated as $3 \sigma/b$, where σ is the standard deviation of the control sample (the sensor incubated in buffer) and b is the slope of the linear part of the calibration plot. The relative standard deviations (RSD%) calculated from triplicate measurements were about 5% for all the concentrations indicating the reproducibility of the assay. It is worth noting that the LOD realized using our biosensor is much lower than the majority of the reported electrochemical biosensors for

Fig. 5 Electrochemical responses of the multiplexed biosensor towards different bacteria for selectivity testing. The SWV responses of the *S. aureus* (a) and *Listeria* (b) biosensors to specific and non-specific proteases. (c) The *S. aureus* biosensor response towards proteases solutions from 10^3 CFU mL⁻¹ of *S. aureus*, *Listeria* and *E. coli*, a mixture of *Listeria* and *E. coli* and a mixture of the three bacteria and BSA protein as control. (d) the *Listeria* biosensor response towards proteases solutions from 10^3 CFU mL⁻¹ of *Listeria*, *S. aureus* and *E. coli*, a mixture of *S. aureus* and *E. coli* and a mixture of the three bacteria and BSA protein as control



S. aureus or *Listeria* [33–45] as shown in Table 1, indicating the ultrasensitivity of the method. Moreover, the simultaneous detection of both bacteria requires very short time (1 min) compared with other reported affinity biosensors [33–45].

Investigation of the selectivity of the array multiplexed biosensor

Several bacterial strains are common food contaminants such as *S. aureus*, *Listeria* and *E. coli*. The selectivity of the array biosensor was tested against BSA as control protein as well as *E. coli* protease. Hence, the biosensor was incubated individually with proteases from *S. aureus*, *Listeria* and *E. coli* for 1 min, and the responses were recorded after pulling away the cleaved magnetic beads. Figure 5 a and b show representative SWV signals of the *S. aureus* and *Listeria* biosensors before and after incubation with specific and non-specific bacterial proteases. Figure 5 c and d show the biosensor responses to BSA, the specific and non-specific bacterial proteases. It can be clearly observed that the BSA did not cause any significant responses on the biosensor. A significant response was only observed with the specific bacterial proteases indicating high selectivity of the biosensor. Moreover, the simultaneous detection of the two bacterial proteases was established by mixing two or three proteases. As shown in the figure, when the mixture contains the specific protease for the immobilized peptide on the sensor chip, significant response was observed, whereas no response detected with other mixtures. These results imply the feasibility of applying our biosensor for multiplexed detection of *S. aureus* and *Listeria* without interference from other non-specific bacteria or matrices. However, the lack of information on the specific proteases for different bacterial strains and their substrates limits the wide applicability of this method for other bacteria. Therefore, extensive screening and specificity studies for peptides for different other bacteria proteases is highly demanded.

Conclusion

In conclusion, we developed a novel array electrochemical biosensor platform for the multiplexed detection of *S. aureus* and *Listeria*. The biosensor was fabricated on gold nanoparticles-modified screen-printed electrodes. Specific peptide/magnetic nanoparticle conjugates for *S. aureus* and *Listeria* were used as substrates for their relevant bacterial proteases. The protease enzyme specific for each bacteria causes a cleavage of the peptide sequence and the release of the magnetic particles from the sensor surface which was exploited for the detection. The electrochemical detection was achieved by measuring the increase in the square wave voltammetry reduction peak current after the peptide/magnetic bead cleavage. The multiplexed biosensor showed

very high sensitivity and selectivity against other non-specific bacterial proteases which commonly contaminate food samples. Moreover, the detection can be achieved in 1 min unlike other affinity-based biosensors which takes much longer time to perform. Thus, we believe that this array biosensor holds great promise for the multiplexed and rapid detection of bacteria to reduce the risk from food poisoning and infection control.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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