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PAPER

A novel electrochemical biosensor for highly selective detection of protease biomarker from *Bacillus licheniformis* with D-amino acid containing peptide

Huiwang Wu, Shucheng Liu, Jianhui Jiang,* Guoli Shen and Ruqin Yu*

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A simple, selective and sensitive electrochemical biosensor has been developed to detect protease biomarker from *Bacillus licheniformis*, a recognized model of the biochemical warfare agent *Bacillus anthracis*. In this assay, the biosensor is constructed using a D-amine acid containing substrate peptide *via* self-assembly of cysteine residual at the C-terminal. A biotin modifier is labelled at the N-terminal of the substrate peptide. This enables sensitive electrochemical detection of the intact substrate peptide using a streptavidin-conjugated alkaline phosphatase, which catalyzes the conversion of electrochemically inactive 1-naphthyl phosphate into electrochemically active phenol. In the presence of the protease biomarker, the peptide is cleaved, and the biotin moiety is removed away from the electrode surface, which results in a decreased electrochemical signal corresponding to the concentration of the protease biomarker. This electrochemical biosensor is simple, sensitive and cost effective. The introduction of D-amino acids into the peptide substrate enables high species selectivity and eliminates the steps for enzyme isolation and purification. Under optimized conditions, the protease can be determined in the concentration range from 0.5 to 100 μg mL^{-1} with a detection limit to 0.16 μg mL^{-1}.

Introduction

Bacteria are a large domain of prokaryotic microorganisms. They are present in most habitats on earth, growing in soil and various aqueous media such as acidic hot springs, living in bodies of plants and animals and different other organic matters.[1,2] Ubiquitous in nature, bacteria include both non-pathogenic and pathogenic species. Some cause serious human diseases as well as damage to human food resources, are misused as potential biological weapons, for example, *Bacillus anthracis* is widely recognized as a biochemical warfare agent because of its ability to cause mortality in humans and long survival period under unfavorable conditions.[3,4] Therefore simple, selective and sensitive methods for the detection of bacteria are critical for effective treatment of pathogenic bacteria infection.

A few methods have been developed for the detection of bacteria. As a common DNA-based technique, polymerase chain reaction (PCR) based approaches are used for detection. PCR identifies pathogen specific DNA sequences information by amplification, with satisfactory accuracy and sensitivity. Several PCR-based methods have been reported for detecting plasmids pXO1 and pXO2 which encode the toxin and capsule, respectively, of *Bacillus anthracis*.[5–9] Despite the many advantages of PCR, it requires sophisticated instrumentation, time consuming sample preparation for the reaction and expertise in molecular biology. Many antibody-based methods have been used extensively to detect bacteria.[10–12] Although immunoreaction has its advantages, the antibody is normally only stable under physiological environment, and its chemical modification often causes a decrease in affinity to targets, limiting its practical applications for antibody detection in the field.

In theory, bacterial enzymes are ideal biomarkers for quick and sensitive identification of microorganisms in clinical samples.[13] Protease is a common enzyme in bacterial secretion and is accessible to detect based on substrate cleavage. There are abundant proteases in organisms, from viruses to humans, making it appropriate for analysis. The cleavage substrate activity is not an exact indicator of a specific pathogen. To overcome this problem substrates, which can specifically distinguish protease(s) from a specific pathogen, need to be developed.[14–17] For example, many protease assays for the detection of *Bacillus anthracis* use fluorescence dye-labelled peptide and fluorescence resonance energy transfer (FRET) technology.[18–20] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is also used to detect the *Bacillus anthracis* protease activity on peptide substrates.[15,21] However, developed substrates used in these methods suffer from a lack of specificity, as they can be digested by a variety of bacterial and human enzymes. Thus, these methods cannot be used to directly detect real samples, because time consuming target enzyme isolation is needed, which hampers development of the detection. Recently, some D-amino acid containing specific peptide substrates have been developed to detect *Bacillus*

State Key Laboratory for Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha, 410082, P. R. China. E-mail: jianhuijiang@hnu.edu.cn; rqyu@hnu.edu.cn; Fax: +86-731-8822782; Tel: +86-731-8822577

species.[22] The presence of D-amino acid is highly specific for bacterial proteases, being abundantly present as a component of bacterial cell walls.[23] D-Amino acid containing substrates can therefore be specifically designed to detect bacterial species.

Electrochemical sensors possess many advantages such as high sensitivity, simple instrumentation, low endogenous background, small analyte volumes, and excellent compatibility with array format and miniaturization. Some electrochemical biosensors for bacteria have also been developed based on antibody and gene probes.[10,26,27] As described above, most are limited by drawbacks. Therefore, in this paper, an electrochemical biosensor was constructed for the detection of the protease biomarker from the *Bacillus licheniformis*, a model of *Bacillus anthracis*. According to a previous report,[22] a D-amino acid containing peptide labelled with biotin is used as the specific substrate. First, the specific peptide is assembled on the gold electrode through the C-terminal Cys interacting with gold. Upon the addition of protease, the substrate is selectively recognized and cleaved. Subsequently, the streptavidin–alkaline phosphatase (SA–ALP) specifically binds the remaining biotin moieties on the electrode surface. ALP can catalyze the conversion of an electrochemically inactive 1-naphthyl phosphate into an electrochemically active phenol, generating an amplified signal for electrochemical readouts.[24–26] Differential pulse voltammetry (DPV) is employed to detect the signal which can then be used to quantify proteases of *Bacillus licheniformis*.

Experimental

Materials and reagents

Protease from *Bacillus licheniformis*, 1-naphthyl phosphate (1-NP), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and 6-mercapto-1-hexanol (MCH) were purchased from Sigma-Aldrich (Shanghai, China). The peptide was synthesized by Sangon Biotech Co. Ltd (Shanghai, China) and had the sequence: biotin-ALNNd-Ld-LKNNLAC. Streptavidin–alkaline phosphatase (SA–ALP) and bovine serum albumin (BSA) were purchased from Beijing Dingguo Biotechnology Company (Beijing, China). All of the other chemicals were of analytical reagent grade and used without further purification. Ultrapure water with an electrical resistance larger than 18.2 MΩ was used throughout.

The phosphate buffer solution (PBS) was a 50 mM K_2HPO_4 solution of pH 7.5. BSA solution (1%, w/v) was prepared with PBS. Washing buffer solution (pH 7.0) consisted of 10 mM tris–HCl. HEPES buffer (20 mM, pH 6.0) containing 10 mM TCEP was used to dissolve peptide.

PBS containing 5 mM $K_3[Fe(CN)_6]$, 5 mM $K_4[Fe(CN)_6]$, and 100 mM KCl was used in electrochemical impedance spectroscopic (EIS) measurements. The measuring medium for the differential pulse voltammetric (DPV) experiments consisted of 100 mM tris–HCl (pH 9.8), 1 mM Mg^{2+} and 4 mM 1-NP.

Apparatus

Differential pulse voltammetric (DPV) and electrochemical impedance spectroscopic (EIS) experiments were carried out on a CHI 760B electrochemical workstation (Shanghai Chenhua Apparatus, Shanghai, China). A conventional three-electrode system contained a gold electrode (2.0 mm diameter) as the working electrode, a platinum foil as the auxiliary electrode, and a saturated calomel electrode (SCE) as the reference. All experiments were performed in a 10 mL voltammetric cell at ambient temperature (25 °C).

Peptide self-assembled on gold electrodes

Gold electrode was polished sequentially with 0.3 μm and 0.05 μm alumina powder, followed by ultrasonic cleaning in ethanol and water. Subsequently, the gold electrode was cleaned with piranha solution (H_2SO_4/H_2O_2, 7:3), and rinsed thoroughly with water. Then, the electrochemical pretreatments were performed by cycling the potential between −0.3 and +1.5 V (*vs.* SCE) in 0.5 M H_2SO_4 solution for 10 min. Finally, the electrode was washed with water and dried under a nitrogen stream.

The gold working electrode was coated with 4 μL of 0.2 mM peptide solution and kept overnight at 4 °C. After the incubation step, the electrode was washed with washing buffer. The unmodified region of the electrode was blocked by immersing the electrode into 1 mM MCH solution for 10 min and then the surface was rinsed with washing buffer.

Protease detection

A series of samples containing different concentrations of protease were prepared in PBS. The peptide-modified electrode was immersed in protease samples and incubated at 37 °C for 1 h. Then 1% BSA solution was dropped onto the electrode and incubated at 37 °C for 30 min to reduce the unspecific adsorption of SA–ALP. Finally, the SA–ALP solution (1 : 100 dilution from the stock solution using 1% BSA solution) was added to the electrode surface and incubated at 37 °C for 40 min.

The electrode modified with the enzyme complex was immersed in 3 mL of measuring medium for 3 min. A DPV voltammogram was recorded at a potential range from 0 to 0.5 V (*vs.* SCE) with a 100 mV s^{-1} scan rate.

Selectivity assay

Escherichia coli (*E. coli*) and *Agrobacterium tumefaciens* were grown overnight at 37 °C. After centrifugation for 10 min at 10 000 rpm, supernatant of the bacteria, containing secreted enzymes, was passed through a 0.22 μm filter. It was then diluted with an equivalent volume of PBS. The human serum and saliva were diluted with equivalent volume of PBS. All samples were used directly, without further purification. The samples were detected in the same way as for protease detection.

Results and discussions

Analytical principle of the biosensor

In order to construct a highly selective biosensor for the detection of protease biomarker, a specific peptide substrate was employed.[22] As is well known, L-amino acids are the major component of natural protein, but D-amino acids are a highly specific component of bacterial cell walls.[28] Bacteria may express

some kinds of protease which can selectively recognize and hydrolyze the D-amino acid-containing substrates. Therefore, D-amino acid-containing substrates can be specifically designed to detect protease biomarker of the target *Bacillus* sp. Herein, a peptide containing two D-leucines is used as the substrate for the detection of protease biomarker from *Bacillus licheniformis*. The presence of the two D-leucines protected the substrate from cleavage by other enzymes and improved the selectivity of the biosensor.

A schematic representation of the analytical principles of the electrochemical biosensor for detection of protease biomarker from *Bacillus licheniformis* is shown in Scheme 1. A self-assembled monolayer of biotin-labelled peptide (red region represents two D-leucines) is formed by strong interaction between the sulfur atom of Cys and the gold surface. The unmodified region of the electrode is blocked by MCH. SA–ALP is added and binds the labelled biotin. ALP catalyzed the conversion of an electrochemically inactive 1-NP into an electrochemically active phenol producing an oxidation peak by electron-transfer reaction. When the sample containing the protease biomarker was added, the peptide was cleaved, the biotin moiety was removed from the electrode surface causing a weaker electrochemical signal that corresponded to the concentration of the protease biomarker.

Fig. 1 shows the DPV curve of the biosensor obtained in response to protease biomarker and in the control experiments, respectively. In the presence of protease, there was a small oxidation peak (curve b in Fig. 1), while in the control experiment, where BSA solution was used instead of protease sample, a very strong current peak (curve a in Fig. 1) was observed. As common enzymes in animal and human, lysozyme and thrombin were investigated for selectivity by the biosensor. Compared to the protease biomarker sample, the recorded oxidation peak of lysozyme (curve c in Fig. 1) and thrombin (curve d in Fig. 1) are clearly higher. These results indicated that the assay for protease biomarker detection can be carried out.

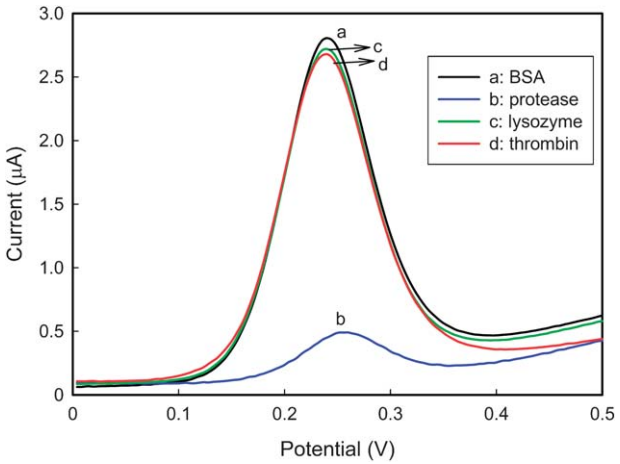
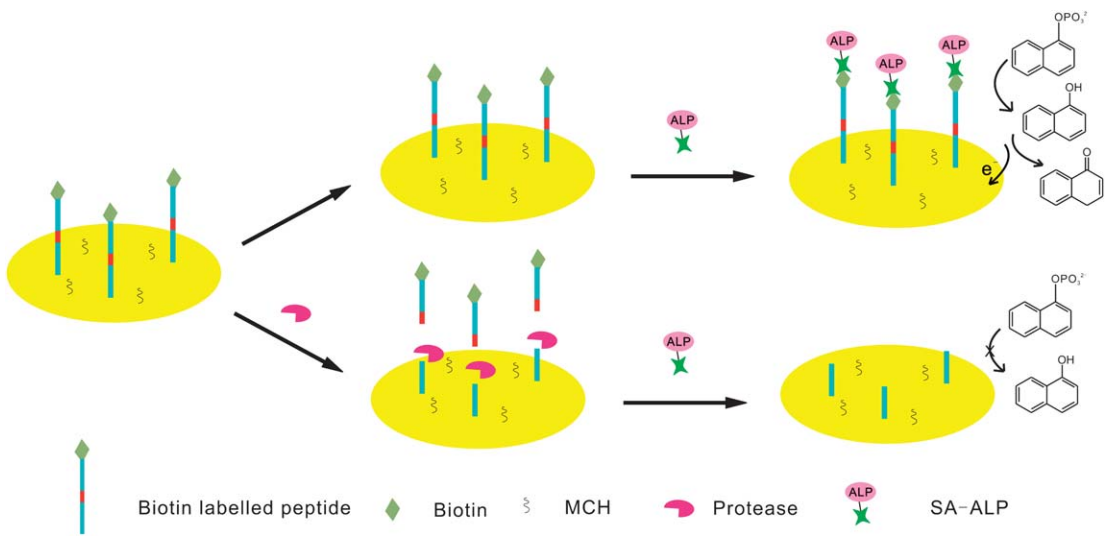


Fig. 1 Differential pulse voltammograms between 0 and 0.5 V *versus* SCE in response to (a) BSA solution; (b) 100 μg mL^{-1} protease; (c) 10 μg mL^{-1} lysozyme; (d) 1 U mL^{-1} thrombin.

Electrochemical characteristics of modified electrode

Electrochemical impedance spectroscopy is a valuable means of probing electrochemical characterization of a surface-modified electrode. As depicted in Fig. 2, starting from the bare gold electrode (curve a), the electrochemical impedance changed sequentially after each step of association. The peptide was assembled on the electrode, and when MCH blocked the unmodified region of the electrode (curve b) impedance increased. Following blocking by BSA the nonspecific site (curve c) impedance also increased. Finally, SA–ALP specifically bound to labelled biotin further increased impedance (curve d). When the peptide-modified electrode was incubated in the protease containing sample, impedance decreased (curve b′). Subsequently, when BSA blocked the nonspecific site (curve c′) impedance increased. Adding SA–ALP did not obviously increase impedance (curve d′). In this section, concentration of



Scheme 1 Schematic illustration of the electrochemical biosensor to detect protease biomarker from *Bacillus licheniformis* using D-amino-containing peptide.

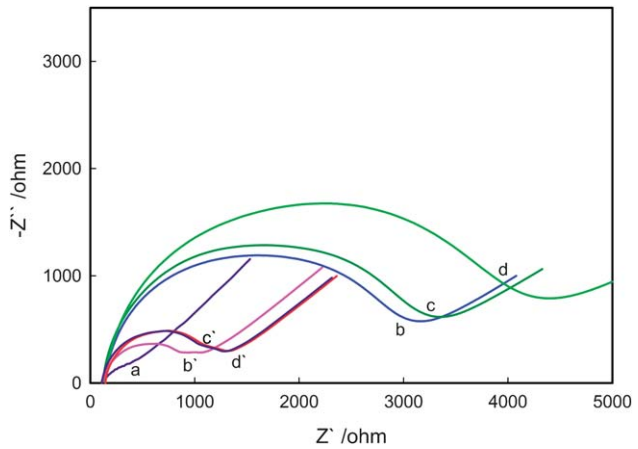


Fig. 2 Nyquist diagrams of electrochemical impedance spectra (in the frequency range of 0.1–10 KHZ) after different steps of modification. (a) Bare gold electrode, (b) peptide/MCH modified electrode, (c) peptide/MCH/BSA modified electrode, (d) peptide/MCH/BSA/SA–ALP modified electrode, (b′) is (b) with added protease, (c′) and (d′) are the same operation as (c) and (d) based on (b′). All measurements were performed in 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution containing 0.1 M KCl.

the protease was 100 μg mL^{-1}. The results indicated that a sensing interface is effectively constructed and successfully applied for the analysis of protease.

Effect of incubation time on the biosensor

The incubation time of protease is critical to the performance of the constructed biosensor. Herein, the incubation time was investigated (Fig. 3). One can observe that the response current decreased with increased incubation time. After 1 h of incubation, the DPV signal decrease reached a plateau. Therefore, 1 h was chosen as the optimal incubation time for protease.

Protease detection

As mentioned above, when a higher concentration of protease is added more peptide is cleaved and less SA–ALP is captured, resulting in a lower current response. The DPV response to different concentrations of protease is shown in Fig. 4A. The DPV current decreased with increasing concentration of protease. Fig. 4B shows the current curve with different protease concentration (0 to 100 μg mL^{-1}). The inserted plot of Fig. 4B is the linear plot of current *versus* logarithmic value of the protease concentration (0.7 to 10 μg mL^{-1}). The regression equation is I (μA) = 1.3329 − 0.7062 × log([protease] (μg mL^{-1})), with R^2 = 0.9954. According to three times standard deviation over blank solution rule, the detection limit was calculated to be 0.16 μg mL^{-1}.

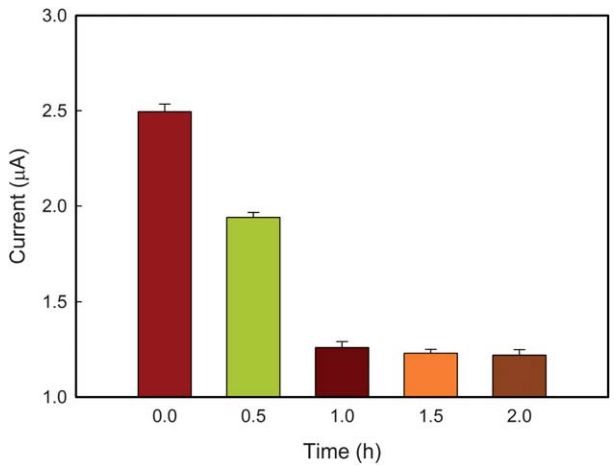


Fig. 3 Peak currents at different incubation times, the concentration of protease is 1 μg mL^{-1}.

Selectivity of the biosensor

In order to evaluate the selectivity of the developed biosensor, interferences from other compounds were investigated, such as human serum, saliva, the secretions of *E. coli* and *Agrobacterium tumefaciens*. As shown in Fig. 5, current signals for all these tested compounds were similar to the blank signal but at a much

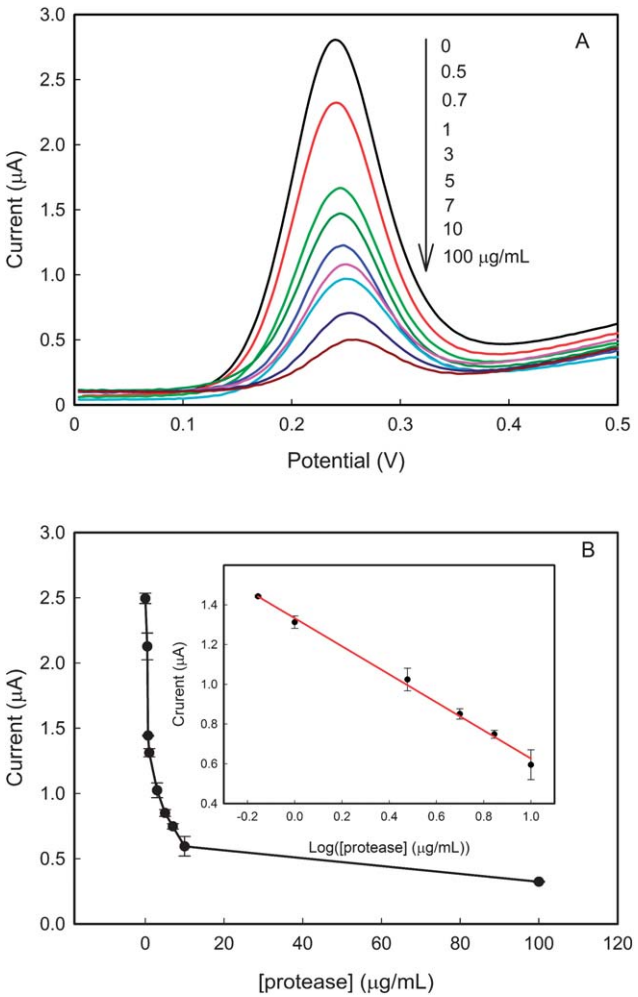


Fig. 4 (A) Differential pulse voltammograms between 0 and 0.6 V *versus* SCE of different concentrations of protease, the curves from top to bottom were 0, 0.5, 0.7, 1, 3, 5, 7, 10, 100 μg mL^{-1}. (B) The response curve obtained with different concentrations from 0 to 100 μg mL^{-1}. The insert shows the calibration curves of protease (0.7–10 μg mL^{-1}). Error bars represent standard deviations (n = 3).

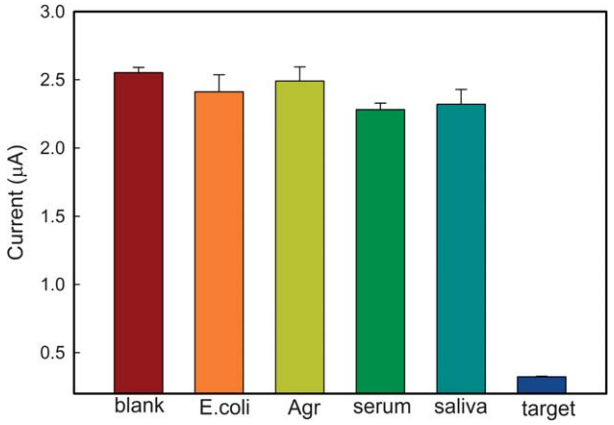


Fig. 5 Selectivity of the biosensor for the secretion of *E. coli* and *Agrobacterium tumefaciens* (Agr), human serum and saliva.

higher level than that for the target protease (100 μg mL^{-1}). These results confirm that the developed strategy has high selectivity for the detection of the protease biomarker from *Bacillus licheniformis*.

Conclusions

In summary, we developed an electrochemical biosensor for the detection of protease biomarker from *Bacillus licheniformis*. This method utilized D-amine acid-containing peptide as the substrate to provide high species selectivity. ALP was employed to generate the amplified DPV current signal, and a detection limit of 0.16 μg mL^{-1} was obtained. Compared with previous reported methods, the proposed method eliminates the steps for enzyme isolation and purification, has the advantages of simplicity, selectivity and sensitivity. This new electrochemical biosensor is expected to have important applications in the development of high-throughput with electrochemical array and potential for bacteria species detection.

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Notes and references

1 G. M. Evans and J. C. Furlong, *Environmental Biotechnology: Theory and Application*, John Willey, West Sussex, 2003.
2 L. M. Prescott, J. P. Harley and D. A. Kleen, *Microbiology*, New York, 5th edn, 2002.
3 T. V. Inglesby, D. A. Henderson, J. G. Bartlett, M. S. Ascher, E. Eitzen, A. M. Friedlander, J. Hauer, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. M. Perl, P. K. Russell and K. Tonat, *JAMA, J. Am. Med. Assoc.*, 1999, **281**, 1735.
4 D. V. Lim, J. M. Simpson, E. A. Kearns and M. F. Kramer, *Clin. Microbiol. Rev.*, 2005, **18**, 583.
5 M. Mock and A. Fouet, *Annu. Rev. Microbiol.*, 2001, **55**, 647.
6 K. A. Edwards, H. A. Clancy and A. J. Baeumner, *Anal. Bioanal. Chem.*, 2006, **384**, 73.
7 M. H. Antwerpen, P. Zimmermann, K. Bewley, D. Frangoulidis and H. Meyer, *Mol. Cell. Probes*, 2008, **22**, 313.
8 D. Volokhov, A. Rasooly, K. Chumakov and V. Chizhikov, *J. Clin. Microbiol.*, 2002, **40**, 4720.
9 S. F. González, M. J. Krug, M. E. Nielsen, Y. Santos and D. R. Call, *J. Clin. Microbiol.*, 2004, **42**, 1414.
10 S. K. Mwilu, A. O. Aluoch, S. Miller, P. Wong and O. A. Sadik, *Anal. Chem.*, 2009, **81**, 7561.
11 R. E. Biagini, D. L. Sammons, J. P. Smith, B. A. MacKenzie, C. A. Striley, J. E. Snawder, S. A. Robertson and C. P. Quinn, *Clin. Vaccine Immunol.*, 2006, **13**, 541.
12 R. Z. Hao, D. B. Wang, X. E. Zhang, G. M. Zuo, H. P. Wei, R. F. Yang, Z. P. Zhang, Z. X. Cheng, Y. C. Guo, Z. Q. Cui and Y. F. Zhou, *Biosens. Bioelectron.*, 2009, **24**, 1330.
13 M. Manafi, W. Kneifel and S. Bascomb, *Microbiol. Rev.*, 1991, **55**, 335.
14 N. D. Rawlings, A. J. Barrett and A. Bateman, *Nucleic Acids Res.*, 2010, **38**, D227.
15 A. E. Boyer, C. P. Quinn, A. R. Woolfitt, J. L. Pirkle, L. G. McWilliams, K. L. Stamey, D. A. Bagarozzi, C. J. Hart Jr and J. R. Barr, *Anal. Chem.*, 2007, **79**, 8463.
16 R. Rasooly, L. H. Stanker, J. M. Carter, P. M. Do, L. W. Cheng, X. H. He and D. L. Brandon, *Int. J. Food Microbiol.*, 2008, **126**, 135.
17 A. Ingram, L. Byers, K. Faulds, B. D. Moore and D. Graham, *J. Am. Chem. Soc.*, 2008, **130**, 11846.
18 R. T. Cummings, S. P. Salowe, B. R. Cunningham, J. Wiltsie, Y. W. Park, L. M. Sonatore, D. Wisniewski, C. M. Douglas, J. D. Hermes and E. M. Scolnick, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 6603.
19 M. Y. Zakharova, N. A. Kuznetsov, S. A. Dubiley, A. V. Kozyr, O. S. Fedorova, D. M. Chudakov, D. G. Knorre, I. G. Shemyakin, A. G. Gabibov and A. V. Kolesnikov, *J. Biol. Chem.*, 2009, **284**, 17902.
20 R. H. Kimura, E. R. Steenblock and J. A. Camarero, *Anal. Biochem.*, 2007, **369**, 60.
21 S. Aravamudhan, P. J. Joseph, Z. Kuklenyik, A. E. Boyer and J. R. Barr, *Conf. Proc. IEEE. Eng. Med. Biol. Soc.*, 2009, 1071.
22 W. E. Kaman, A. G. Hulst, P. T. W. van Alphen, S. Roffel, M. J. van der Schans, T. Merkel, A. van Belkum and F. J. Bikker, *Anal. Chem.*, 2011, **83**, 2511.
23 J. V. Höltje, *Microbiol. Mol. Biol. Rev.*, 1998, **62**, 181.
24 R. M. Pemberton, J. P. Hart, P. Stoddard and J. A. Foulkes, *Biosens. Bioelectron.*, 1999, **14**, 495.
25 M. L. Del Giallo, F. Lucarelli, E. Cosulich, E. Pistarino, B. Santamaria, G. Marrazza and M. Mascini, *Anal. Chem.*, 2005, **77**, 6324.
26 G. Carpini, F. Lucarelli, G. Marrazza and M. Mascini, *Biosens. Bioelectron.*, 2004, **20**, 167.
27 J. Wang, G. Rivas and X. H. Cai, *Electroanalysis*, 1997, **9**, 395.
28 G. D. Shockman, L. Daneo-Moore, R. Kariyama and O. Massidda, *Microb. Drug Resist.*, 1996, **2**, 95.