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2011

Cheng, M. S., Lau, S. H., Chow, V. T. K., & Toh, C. S. (2011). Membrane-based electrochemical nanobiosensor for escherichia coli detection and analysis of cells viability. *Environmental science & technology*, 45(15), 6453-6459.

<https://hdl.handle.net/10356/93998>

<https://doi.org/10.1021/es200884a>

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Membrane-Based Electrochemical Nanobiosensor for *Escherichia coli* Detection and Analysis of Cells Viability

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ABSTRACT

A sensitive and selective membrane-based electrochemical nanobiosensor is developed for specific quantitative label-free detection of *Escherichia coli* (*E. coli*) cells and analysis of viable but nonculturable (VBNC) *E. coli* cells which remain mostly undetected using current methods. The sensing mechanism relies on the blocking of nanochannels of a nanoporous alumina-membrane modified electrode, upon the formation of immune complexes at the nanoporous membrane. The resulting obstacle to diffusive mass transfer of a redox probe in the analysis solution to the underlying platinum electrode reduces the Faradaic signal response of the biosensor, measured using cyclic voltammetry. Antibody loading under conditions of varying antibody concentrations and pHs are optimized. The biosensor gives a low detection limit of 22 cfu mL⁻¹ ($R^2 = 0.999$) over a wide linear working range of 10 to 10⁶ cfu mL⁻¹. It is specific

towards *E. coli* with minimal cross-reactivity to two other pathogenic bacteria (commonly found in waters). Relative standard deviation (RSD) for triplicate measurements of 2.5 % indicates reasonably useful level of reproducibility. Differentiation of live, VBNC and dead cells are carried out after the cell capture and quantitation step, by simple monitoring of the cells' enzyme activity using the same redox probe in the analysis solution, in the presence of glucose.

KEYWORDS

E. coli, electrochemistry, nanobiosensor, nanoporous, membrane, bacteria, detection, viable but nonculturable (VBNC)

1. INTRODUCTION

In recent times, methods for the detection of microbial species have attracted great interest because of the urgent needs to identify and study disease-causing microbes arising from contamination in water and food sources, terrorism acts or widespread proliferation under favorable environmental conditions. Every year, 1.5 million deaths are caused by pathogens in drinking water and food sources.¹ One highly relevant and commonly targeted indicator for routine analysis of contaminated water and food sources is the bacterium *Escherichia coli*.² Though most strains of *E. coli* are harmless, the presence of *E. coli* in water or food indicates deterioration of microbiological water quality, fecal contamination and possible existence of pathogenic microbial species including *E. coli*. Conventional methods for the detection of *E. coli* include plate count, membrane filter and multiple tube fragmentation.³ These usually involve selective culturing and isolation of cells followed by biochemical and serological confirmations which are reliable and highly accurate but often time consuming, multiple-steps procedure, lacks

specificity and are labor intensive.

Detection methods using nanoporous membranes⁴⁻⁷ have been reported for the sensing detection of drugs, proteins and oligonucleotides.⁵ For example, a real-time biosensor based on morpholino-modified porous anodic alumina membrane has been demonstrated for label-free detection of the interaction between DNA and nanochannels,⁸ based on electrostatic obstruction of the diffusion of redox species, ferricyanide detected by gold film electrochemical detector sputtered at the end of nanochannels. A simple label-free electrochemical biosensor based on screen-printed carbon electrode (SPCE) modified with alumina membrane has been developed for the detection of protein.⁹ Blocking of the pores upon immunological interaction with antibody functionalized nanochannels is probed by differential pulse voltammetry (DPV) using redox species, ferricyanide.

Previously, it was presumed that cells are dead when unable to grow on routine bacteriological media. In many circumstances, these cells are alive and can regain normal metabolic activity upon resuscitation during this latent phase, now known as the viable but nonculturable (VBNC) state.¹⁰ Pathogenic bacteria in VBNC state constitute an important reservoir for infection which can evade detection by many conventional methods.¹¹ Therefore, one major challenge for accurate detection of bacteria population number is the inability to differentiate between dead, latent and live bacteria.

Several methods for physiological characterization of bacteria include mRNA detection using reverse transcriptase-PCR,¹² sol-gel immobilization,¹³ direct viable count,¹⁴ double staining method using epifluorescence microscopy,¹⁵ and flow cytometry.¹⁶ Electrochemical monitoring of rate of cell metabolism such as respiration has been reported¹⁷ which can provide an alternative rapid way for identifying VBNC cells and improve the accuracy of quantitating bacterial cells.

Recently, electrodes placed closed to cell surfaces can directly measure reactive oxygen species (ROS) and reactive nitrogen species (RNS) secreted by macrophage.¹⁸ Another useful approach is to monitor oxidation or reduction currents generated by artificially added redox species such as $\text{Fe}(\text{CN})_6^{4-}$ which can function as alternative electron acceptor to oxygen for cell processes. $\text{Fe}(\text{CN})_6^{4-}$ concentration level near the cell surface can reveal interesting information on toxic effect of Ag^+ ion on *E. coli* cells.¹⁷ In another report, $\text{Fe}(\text{CN})_6^{4-}$ was used to probe biochemical oxidations of glucose and acetate in *E. coli* cells under anaerobic condition.¹⁹

Herein, we report the strategy to detect *E. coli* cells by monitoring the nanoporous membrane-based biosensor's Faradaic current response towards a redox species, which is sensitive towards the formation of immunocomplexes which block the movement of the redox species. Previously, such biosensors have shown ultrasensitivity for protein and whole virus by monitoring the Faradic current of ferrocene species at the membrane-electrode interface.^{20, 21} Finally, we report the first demonstration of using the same biosensor to assess viability of these cells by simple addition of glucose, followed by the monitoring of the current derived from bacterial enzyme reaction mediated by ferrocene species already present in the analysis solution.

2. EXPERIMENTAL SECTION

2.1. Fabrication of nanoporous alumina membrane-modified platinum electrode. Home-made platinum wire electrode was fabricated using micropipette tip, epoxy glue, copper wire and platinum wire (76 μm diameter). The electrode tip was polished with 1.0 μm followed by 0.3 μm diameter alumina powder. By using 99.999% purity aluminium target and Denton discovery 18 sputtering system with sputtering power of 100 W in an atmosphere of research-grade Argon at low pressure of 5×10^{-3} Torr, aluminium films with thickness in the range of 0.3-0.5 μm were

sputter-coated onto the surface of electrode tip. All electrodes were rinsed with ultrapure water. Anodization was performed using Apelex electrophoresis power supply model P304 minipac II. Anodization using the surface contact anodization method was described elsewhere.²² Etching in 3% H₃PO₄ for 15 min was conducted in order to broaden the size of alumina nanopore channels. Longer etching time is not desirable as it would cause membrane disintegration. All potentials were measured with respect to the silver-silver chloride (saturated KCl) reference electrode.

2.2. Construction of electrochemical biosensor and *E. coli* analysis steps. Prior to immobilization of antibodies, etched alumina membrane-modified electrode was cleaned with ultra-pure water and air-dried. 10 μ L of 0.1 mg mL⁻¹ anti-*E. coli* antibodies was applied onto the alumina membrane-modified electrode tip and stored at 4 °C overnight. The unbound antibodies were removed by rinsing with 0.1 M buffered saline, pH 6.8. The antibody-immobilized electrode was subsequently treated with 1% (w/v) bovine serum albumin (BSA) in 0.1 M phosphate buffered saline, pH 6.8 at room temperature for 1 h to block the nonspecific binding sites. After rinsing with 0.1 M phosphate buffered saline, pH 6.8, the biosensor was ready for use. A three-electrode system was used for the measurement of electrochemical response towards redox probe ferrocenemethanol in the analysis solution, using the biosensor as working electrode (76 μ m diameter Pt disk embedded in ~0.3 cm diameter inert epoxy sheath), Pt wire mesh as auxiliary electrode and silver/silver chloride (saturated KCl) as reference electrode. 10-Fold serial dilutions of pure culture of *E. coli* from 10⁷ to 10 cfu mL⁻¹ were prepared in 0.1 M phosphate buffered saline, pH 6.8. Detection of *E. coli* was conducted by applying 10 μ L of *E. coli* solutions with different cell numbers onto the biosensor surface and incubated at 37 °C for 1 h. The biosensor was then rinsed with 0.1 M phosphate buffered saline, pH 6.8 to remove the

nonspecifically bound proteins and *E. coli*. Subsequently, the biosensor was conditioned using 10 repetitive potential cycles at 50 mV s^{-1} from 0 to 0.6 V in the analysis solution containing redox probe.

2.3 Probing the activity of live, dead and VBNC *E. coli*. This is carried out by probing the rate of enzyme catalytic oxidation of glucose using ferrocenemethanol as redox probe using a batch system in which the biosensor with captured *E. coli* cells was kept immersed in 25 mL of 1.0 mM ferrocenemethanol in 0.1 M phosphate buffered saline, pH 6.8. Concentration of glucose in the solution was varied by adding aliquots of 1.0 M glucose stock solution containing 1 mM ferrocenemethanol. Solution was mixed thoroughly prior to each measurement. Cyclic voltammetry at glucose concentrations of 10, 20, 40, 60 and 80 mM were recorded for live *E. coli* in 0.1 M phosphate buffered saline, pH 6.8 and 1.0 mg L^{-1} chlorine solution. Control study using heat-killed *E. coli* was conducted using the same procedures stated above. See Supporting Information for reagents, equipment and other procedures.

Scheme 1

3. RESULTS AND DISCUSSION

3.1. Membrane-based electrochemical nanobiosensor. Scheme 1 shows the design of operation of the biosensor. Anti-*E. coli* antibody is physically immobilized onto the membrane surface and channel walls of alumina nanopores followed by backfilling of remaining empty sites using BSA to block nonspecific binding. The biosensor is rinsed with copious amount of phosphate buffered saline, pH 6.8 followed by potential cycling between 0 and 0.6 V (vs.

Ag/AgCl) in 1 mM ferrocenemethanol, 0.1 M phosphate buffered saline, pH 6.8 to obtain stable voltammetric response. The fabricated biosensor is subsequently incubated with a solution containing *E. coli* cells, followed by measurements of cyclic voltammetry signal in the presence of the redox probe, ferrocenemethanol. Previously, it was revealed the signal response of the same membrane-based nanobiosensor towards virus and protein depends on amount of redox species moving through the membrane nanochannels.^{20, 21} Figure 1 shows that *E. coli* cells can also block the pores which results in a change of the biosensor signal response.

Figure 1

Figure 1A shows the typical cyclic voltammograms obtained at the membrane electrode after each step of the biosensor construction procedure, in a ferrocenemethanol solution. As expected, the voltammetric peak current increases after etching due to enlargement of the pore openings and nanochannel diameters. Subsequent immobilization of antibody and BSA within the membrane nanochannels, reduces the cross-sectional areas of the membrane nanochannels and impedes movement of the redox species.^{20, 21} A significant decrease in the peak current is clearly observed before and after 1 h incubation of the biosensor with 10^6 cfu mL⁻¹ *E. coli* cells at 37 °C. Because of the structural variation of the porous membrane layers, the voltammetric peak current response of the biosensors towards 10^6 cfu mL⁻¹ *E. coli* range between 15.5 and 18.4 nA for 3 different alumina electrodes. As opposed to this, signal responses ($I_{E.coli}$) when normalized against signal response derived from the same biosensor after antibody immobilization in the absence of cells ($I_{E.coli=0}$) give excellent relative standard error of 2.5%. This observation is consistent with previous work on membrane-based protein and virus

biosensors and indicates normalization of signal responses against blank signal (after antibody and BSA immobilization) from the same biosensor significantly improves precision and reduces need for calibration of individual biosensors from same batch of construction.

Normalized signal response of a platinum electrode without the membrane overlayer but surface coated with immobilized antibody and BSA, gives relatively unchanged signal response at increasing *E. coli* concentration (See Figure S2-S1 in Supporting Information). In contrast, the biosensor gives the typical decreasing normalized signal response which is linear in relation to the logarithm of *E. coli* concentration. This clearly indicates the membrane overlayer is critical for sensitive biosensor performance during *E. coli* detection. In another control experiment, a biosensor prepared without BSA, also shows response towards *E. coli* but of slightly lower sensitivity (gentler slope). A further control using a biosensor prepared from unetched alumina membrane-modified electrode with pore sizes $\sim 10\text{-}30\text{ nm}^{20}$, does not respond towards *E. coli* at concentrations below 10^5 cfu mL^{-1} unlike the etched alumina membrane-modified electrodes. The poorer working range of the biosensor prepared from unetched alumina membrane-modified electrode is attributed to the presence of remnant alumina barrier layer which reduces the heterogeneous rate constant of redox probe at the sensing electrode surface.²²

Figure 2

3.2. Biosensor optimization. Performance of the membrane-based nanobiosensor relies on two critical parameters, the antibody loading and the nanochannel dimensions that influence movement of the redox species. In the following, the biosensor signal response is optimized with regard to the antibody concentration and pH during biosensor construction. These are expected to

influence the capture of *E. coli* cells and the nanochannel dimensions. Figure 2A, C reveals the effect of antibody concentration and pH on the loading amount of antibody on the membrane-based biosensor. The loading amount is measured from the change in the biosensor's signal response before and after antibody loading. The signal response towards ferrocenemethanol after antibody immobilization is normalized to the signal response of the same biosensor in the absence of antibody, where no change in normalized signal response (100%) indicates zero antibody loading. The consequent sensitivities of the biosensors prepared under these conditions are measured from the slopes of normalized biosensor responses after incubation in varying *E. coli* concentration (Figure 2B, D).

By doubling the antibody concentration from 0.05 to 0.1 mg mL⁻¹, the biosensor response slope (or sensitivity) derived from the logarithmic plots increases by ~1.5 times. Change in sensitivity is minimal when concentration of antibody used to prepare the biosensor is larger than 0.2 mg mL⁻¹ (Figure 2B). The enhanced sensitivity follows the trend of increased antibody loading (Figure 2A) which gives higher biosensor signal response at the increased antibody concentration. An intermediate antibody concentration of 0.1 mg mL⁻¹ is selected as the optimal condition so low detection limit and wide linear detection range can be derived. In addition, effect of pH on antibody loading and biosensor signal response is examined over 5 different pH values (Figure 2C and D). Antibody loading increases when pH is increased from 3.8 to 6.8, but decreases at higher pHs of 8.3 and 9.8. Maximum antibody loading occurs at pH 6.8 (Figure 2C), about midway between known neutral pH of alumina at ~7.2²³ and the isoelectric point of rabbit anti-*E. coli* antibody at ~pHs 6.1-6.5.²⁴ This trend compares favorably with previous reported membrane-based virus biosensor using IgM antibody instead of IgG, indicates similar interactions between alumina surface and antibody which are largely electrostatic with negligible

hydrophobic contributions.²¹ In general, the trend in biosensor sensitivity correlates with the change in antibody loading. However, at the extreme pHs 3.8 and 9.8, the biosensor sensitivity shows significant deviation from intermediary pHs. This suggests highly probable structural and charge variations of the antibody protein could also influence its binding affinity for *E. coli* cells.²⁵

3.3 Analytical performance of the biosensor. The voltammetric peak currents derived from the biosensor, decrease in the presence of increasing concentration of *E. coli* ranging from 0 to 10^7 cfu mL⁻¹ (See Figure S2-S2 in Supporting Information). The normalized biosensor signal response is logarithmically linear from 10 to 10^6 cfu mL⁻¹ *E. coli* (Figure 1B) with extremely low detection limit of 22 cfu mL⁻¹ *E. coli*. The detection limit is determined from the minimum *E. coli* cell concentration which gives a signal reduction equivalent to three times the standard deviation of the signal in the absence of *E. coli* cell (see Supporting Information for calculation of detection limit). Other highly attractive features of this biosensor are the simple procedure using *E. coli* cells and antibodies with rapid analysis time of ca. 90 min which is much shorter than conventional ELISA method (6-7 h).²⁶ It involves label free detection which avoids the need of additional labeling procedure such as utilization of fluorescent dye-labeled²⁷ or enzyme conjugated²⁸ secondary antibody. These analytical performance exceeds those using surface plasmon resonance (SPR),²⁹ enzyme-linked immunosorbent assay (ELISA),²⁶ fiber optic,²⁷ and piezoelectric based on quartz crystal microbalance (QCM)³⁰ methods which, in general, give detection limits in the range of 10^2 to 10^4 cfu mL⁻¹. The biosensor detection limit is comparable to recent biosensors which monitor redox products derived from enzyme reactions of *E. coli* cells^{31, 32} and high sensitivity impedimetric immunosensor.³³ In addition, three different

biosensors prepared under identical conditions were found to give relatively constant and excellent RSD of 0.6-2.5 % over five orders of magnitude change in *E. coli* concentration.

Figure 3

Selective detection of *E. coli* is necessary for accurate measurement of coliform bacteria which are excellent indicator for fecal contamination and water quality. Additionally, high specificity biosensor performance can be extremely useful for unambiguous quantitative determination of particular pathogenic bacteria species. Therefore, we evaluate the specificity of this biosensor for *E. coli* in comparison with its response towards two other Gram-negative bacteria, *Serratia marcescens* (*S.marcescens*) and *Salmonella typhimurium* (*S. typhimurium*). Figure 3 shows the normalized biosensor signal responses of three biosensors prepared with anti-*E. coli* antibody and after 1 h incubation with bacteria species over four concentrations (10^3 , 10^4 , 10^5 and 10^6 cfu mL⁻¹). At 10^6 cfu mL⁻¹, the changes in biosensor signal responses towards *S. marcescens* and *S. typhimurium* constitute 22.6-28.4% of the biosensor signal change in the presence of *E. coli*. At 10^3 cfu mL⁻¹, interferences from these nonspecific species are significantly lower at 8.2-12.4 % of signal change towards *E. coli*. In contrast, several immunosensing methods for *E.coli* determination suffer severe interferences (up to 50%) from non-specific bacteria species.^{26, 34, 35} The higher specificity of the membrane-based biosensor towards *E. coli* compared to *S. marcescens* and *S. typhimurium* is highly relevant to the determination of *E.coli* in surface water sources where typical concentrations of bacteria species are 6-1000 cells mL⁻¹(*E. coli*) and 0.001-58 cells mL⁻¹ (*Salmonella*).³⁶

3.4. Cell viability measurements. In this work, we add neutral redox species, ferrocenemethanol, and glucose to the cell solution, in order to probe activities of redox enzymes D-glucose dehydrogenase (GDH) on the outer surface of cytoplasmic membrane of *E. coli* cells captured on the biosensor membrane. GDH can oxidize D-glucose to D-gluconolactone within the periplasmic space,³⁷ using ferrocenemethanol (FcMeOH) as the artificial electron acceptor. Channel proteins present in the outer cell membrane allow free diffusion of low-molecular weight species ($MW \leq 1000$) into the periplasmic space.³⁸ Thus, oxidized and reduced forms of ferrocenemethanol can freely diffuse between the enzymes sites at the cytoplasmic membrane and the membrane electrode and mediate the electron transfer as follows:³⁷

Figure 4

Figures 4A, B and C represent the respective linear sweep voltammetric responses of live, VBNC and dead *E. coli* upon successive additions of glucose in 1 mM ferrocenemethanol. *E. coli* cells in VBNC state were derived from live cells after immersion in solution containing 1.0 mg L⁻¹ chlorine.³⁹ Figure 4A and B shows increases in biosensor signal responses towards ferrocenemethanol in the presence of glucose for live and VBNC *E. coli* cells respectively. These increases in current are attributed to redox cycling of ferrocenemethanol by active GDH enzyme on the outer surface of cytoplasmic membrane, in the presence of glucose. In the potential range from 0.24 to 0.28 V (vs. Ag/AgCl) between the cathodic and anodic peaks, the biosensor is most sensitive towards the viability state of the cell. This is attributed to the

coupling of the enzyme-glucose reaction at the cytoplasmic membrane to the redox species reaction at the electrode surface, thus changes in the enzyme kinetics can affect the heterogeneous and mass transfer kinetics which are limiting in this potential range. From 0.24 to 0.28 V (vs. Ag/AgCl), the changes in current responses for live and VBNC *E. coli* cells upon consecutive additions of 10 and 80 mM glucose are larger than the RSD of 1.4% for ten repetitive potential cycling at the same biosensor. In contrast, control experiment using heat-killed *E. coli* revealed small decreasing signal responses (Figure 4C). Figure 4D shows normalized biosensor response of live and VBNC *E. coli* towards glucose addition. Non-linear curve fitting of these catalytic current responses toward ferrocenemethanol in the presence of glucose, normalized to blank signals, give apparent Michaelis-Menten constant, K_m (D-glucose) value of 7.1 ± 1.2 mM and 45.3 ± 20.7 mM for live and VBNC *E. coli* respectively (Figure 4D). The proximity between apparent K_m value of glucose enzyme in live *E. coli* and reported K_m value of GDH enzyme (2.1 ± 0.2 mM) suggests this method is highly relevant toward measurement of GDH enzyme activity in live *E. coli* cells.⁴⁰ The larger apparent K_m value observed in VBNC *E. coli* is likely due to either lower enzyme activity which requires larger amount of glucose substrate to reach maximum enzymatic rate or limitation by transport of glucose into the cell.

In conclusion, the nanoporous membrane-based electrochemical biosensor outperforms present immunoassay methods, is highly suitable for sensitive and selective detection of *E. coli*. It can achieve a low detection limit of 22 cfu mL^{-1} with 5 orders of magnitude linear range from 10 to 10^6 cfu mL^{-1} , extremely short analysis time of 90 min and distinctly identify *E. coli* from two other bacteria species at high concentration of 10^6 cfu mL^{-1} . Potential uses of the biosensor include on-site water quality measurements in near future works and development of specific

biosensors for wide range of other bacteria species where specific binding antibody can be isolated. Further, nonculturable *E. coli* cells induced into the VBNC state such as in some treated waters can be potentially differentiated from dead and live cells as demonstrated in this work using the same biosensor design.

ACKNOWLEDGEMENTS

The authors thank NTU for research grant, PhD research scholarship for C.M.S., laboratory staff from NUS Department of Microbiology for spattering and Thong Khar Tiang Nee Chan for providing *S. marcescens* and *S. typhimurium*.

SUPPORTING INFORMATION AVAILABLE

Detailed materials and methods section, detection limit calculation, normalized biosensor signal responses of bare platinum electrode, electrode coated with unetched membrane, electrode coated with etched membrane but without BSA and electrode coated with etched membrane and adsorbed BSA obtained for various control studies (S2-S1), cyclic voltammograms of an etched alumina membrane-modified electrode with increasing concentrations of *E. coli* (S2-S2).

REFERENCES

- (1) Prüss-Üstün, A.; Bos, R.; Gore, F.; Bartram, J., *Safer water, better health: costs, benefits and sustainability of interventions to protect and promote health*. World Health Organization: Geneva, 2008.
- (2) Edberg, S. C.; Rice, E. W.; Karlin, R. J.; Allen, M. J., *Escherichia coli*: the best biological drinking water indicator for public health protection. *J. Appl. Microbiol.* **2000**, *88*, 106S-116S.
- (3) Clesceri, L.; Greenberg, A.; Eaton, A., *Standard Methods of the Examination of Water and Wastewater*, 20th ed.; American Public Health Association, American Water Works Association, Water Environment Federation: Washington, DC, 1998.
- (4) Daniele, S.; Bragato, C.; Battiston, G. A.; Gerbasi, R., Voltammetric characterisation of Pt-TiO₂ composite nanomaterials prepared by metal organic chemical vapour deposition (MOCVD). *Electrochim. Acta* **2001**, *46* (19), 2961-2966.
- (5) Martin, C. R.; Siwy, Z. S., Learning nature's way: Biosensing with synthetic nanopores. *Science* **2007**, *317* (5836), 331-332.
- (6) McKenzie, K. J.; Marken, F.; Hyde, M.; Compton, R. G., Nanoporous iron oxide membranes: layer-by-layer deposition and electrochemical characterisation of processes within nanopores. *New J. Chem.* **2002**, *26* (5), 625-629.
- (7) Zairi, S.; Martelet, C.; Jaffrezic-Renault, N.; Vocanson, F.; Lamartine, R.; M'Gaieth, R.; Maaref, H.; Gamoudi, M., p-type porous-silicon transducer for cation detection: effect of the porosity, pore morphology, temperature and ion valency on the sensor response and generalisation of the Nernst equation. *Appl. Phys. A-Mater. Sci. Process.* **2001**, *73* (5), 585-593.
- (8) Li, S. J.; Li, J.; Wang, K.; Wang, C.; Xu, J. J.; Chen, H. Y.; Xia, X. H.; Huo, Q., A nanochannel array-based electrochemical device for quantitative label-free DNA analysis. *ACS Nano* **2010**, *4* (11), 6417-6424.
- (9) Escosura-Muniz, A.; Merkoci, A., Label-free voltammetric immunosensor using a nanoporous membrane based platform. *Electrochem. Commun.* **2010**, *12* (6), 859-863.
- (10) Oliver, J. D., The viable but nonculturable state in bacteria. *J. Microbiol.* **2005**, *43*, 93-100.
- (11) Passerat, J.; Got, P.; Dukan, S.; Monfort, P., Respective roles of culturable and viable-but-nonculturable cells in the heterogeneity of *Salmonella enterica* serovar typhimurium invasiveness. *Appl. Environ. Microbiol.* **2009**, *75* (16), 5179-5185.
- (12) Oliver, J. D., Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* **2010**, *34* (4), 415-425.
- (13) Kuncova, G.; Podrazky, O.; Ripp, S.; Trogl, J.; Sayler, G. S.; Demnerova, K.; Vankova, R., Monitoring of the viability of cells immobilized by sol-gel process. *J. Sol-Gel Sci. Technol.* **2004**, *31* (1-3), 335-342.
- (14) Besnard, V.; Federighi, M.; Cappelletti, J. M., Development of a direct viable count procedure for the investigation of VBNC state in *Listeria monocytogenes*. *Lett. Appl. Microbiol.* **2000**, *31* (1), 77-81.
- (15) Delgado-Viscogliosi, P.; Simonart, T.; Parent, V.; Marchand, G.; Dobbelaere, M.; Pierlot, E.; Pierzo, V.; Menard-Szczebara, F.; Gaudard-Ferveur, E.; Delabre, K.; Delattre, J. M., Rapid method for enumeration of viable *Legionella pneumophila* and other *Legionella* spp. in water. *Appl. Environ. Microbiol.* **2005**, *71* (7), 4086-4096.
- (16) Khan, M. M. T.; Pyle, B. H.; Camper, A. K., Specific and rapid enumeration of viable but

- nonculturable and viable-culturable Gram-negative bacteria by using flow cytometry. *Appl. Environ. Microbiol.* **2010**, 76 (15), 5088-5096.
- (17) Holt, K. B.; Bard, A. J., Interaction of silver(I) ions with the respiratory chain of *Escherichia coli*: An electrochemical and scanning electrochemical microscopy study of the antimicrobial mechanism of micromolar Ag. *Biochemistry* **2005**, 44 (39), 13214-13223.
 - (18) Amatore, C.; Arbault, S.; Koh, A. C. W., Simultaneous detection of reactive oxygen and nitrogen species released by a single macrophage by triple potential-step chronoamperometry. *Anal. Chem.* **2010**, 82 (4), 1411-1419.
 - (19) Wang, Y. F.; Cheng, S. S.; Tsujimura, S.; Ikeda, T.; Kano, K., *Escherichia coli*-catalyzed bioelectrochemical oxidation of acetate in the presence of mediators. *Bioelectrochemistry* **2006**, 69 (1), 74-81.
 - (20) Koh, G.; Agarwal, S.; Cheow, P. S.; Toh, C. S., Development of a membrane-based electrochemical immunosensor. *Electrochim. Acta* **2007**, 53 (2), 803-810.
 - (21) Nguyen, B. T. T.; Koh, G.; Lim, H. S.; Chua, A. J. S.; Ng, M. M. L.; Toh, C. S., Membrane-based electrochemical nanobiosensor for the detection of virus. *Anal. Chem.* **2009**, 81 (17), 7226-7234.
 - (22) Koh, G.; Agarwal, S.; Cheow, P. S.; Toh, C. S., Characterization of the barrier layer of nanoporous alumina films prepared using two different contact configurations. *Electrochim. Acta* **2007**, 52 (8), 2815-2821.
 - (23) Todorovic, Z. N.; Milonjic, S. K.; Dondur, V. T., The influence of the solid/liquid ratio on the point of zero charge of alumina. In *Progress in Advanced Materials and Processes*, Uskokovic, D. P.; Milonjic, S. K.; Rakovic, D. I., Eds. Trans Tech Publications Ltd: Zurich-Uetikon, 2004; Vol. 453-454, pp 361-366.
 - (24) Wu, X. Z.; Huang, T. M.; Mullett, W. M.; Yeung, J. M.; Pawliszyn, J., Determination of isoelectric point and investigation of immunoreaction in peanut allergenic proteins-rabbit IgG antibody system by whole-column imaged capillary isoelectric focusing. *J. Microcolumn Sep.* **2001**, 13 (8), 322-326.
 - (25) Wright, G. G.; Schomaker, V., Studies on the denaturation of antibody . III. Kinetic aspects of the inactivation of diphtheria antitoxin by urea. *J. Am. Chem. Soc.* **1948**, 70 (1), 356-364.
 - (26) Wang, N.; He, M.; Shi, H. C., Novel indirect enzyme-linked immunosorbent assay (ELISA) method to detect Total *E-coli* in water environment. *Anal. Chim. Acta* **2007**, 590 (2), 224-231.
 - (27) Geng, T.; Uknalis, J.; Tu, S. I.; Bhunia, A. K., Fiber-optic biosensor employing Alexa-Fluor conjugated antibody for detection of *Escherichia coli* O157 : H7 from ground beef in four hours. *Sensors* **2006**, 6 (8), 796-807.
 - (28) Lin, Y. H.; Chen, S. H.; Chuang, Y. C.; Lu, Y. C.; Shen, T. Y.; Chang, C. A.; Lin, C. S., Disposable amperometric immunosensing strips fabricated by Au nanoparticles-modified screen-printed carbon electrodes for the detection of foodborne pathogen *Escherichia coli* O157 : H7. *Biosensors & Bioelectronics* **2008**, 23 (12), 1832-1837.
 - (29) Oh, B. K.; Kim, Y. K.; Bae, Y. M.; Lee, W. H.; Choi, J. W., Detection of *Escherichia coli* O157 : H7 using immunosensor based on surface plasmon resonance. *J. Microbiol. Biotechnol.* **2002**, 12 (5), 780-786.
 - (30) Su, X. L.; Li, Y. B., A self-assembled monolayer-based piezoelectric immunosensor for rapid detection of *Escherichia coli* O157 : H7. *Biosens. Bioelectron.* **2004**, 19 (6), 563-574.
 - (31) Cheng, Y. X.; Liu, Y. J.; Huang, J. J.; Li, K.; Xian, Y. Z.; Zhang, W.; Jin, L. T., Amperometric tyrosinase biosensor based on Fe₃O₄ nanoparticles-coated carbon nanotubes

- nanocomposite for rapid detection of coliforms. *Electrochim. Acta* **2009**, 54 (9), 2588-2594.
- (32) Yang, Q. Y.; Liang, Y.; Zhou, T. S.; Shi, G. Y.; Jin, L. T., Electrochemical investigation of platinum-coated gold nanoporous film and its application for *Escherichia coli* rapid measurement. *Electrochem. Commun.* **2009**, 11 (4), 893-896.
 - (33) Escamilla-Gomez, V.; Campuzano, S.; Pedrero, M.; Pingarron, J. M., Gold screen-printed-based impedimetric immunobiosensors for direct and sensitive *Escherichia coli* quantisation. *Biosens. Bioelectron.* **2009**, 24 (11), 3365-3371.
 - (34) Geng, P.; Zhang, X. N.; Meng, W. W.; Wang, Q. J.; Zhang, W.; Jin, L. T.; Feng, Z.; Wu, Z. R., Self-assembled monolayers-based immunosensor for detection of *Escherichia coli* using electrochemical impedance spectroscopy. *Electrochim. Acta* **2008**, 53 (14), 4663-4668.
 - (35) Zhang, X. N.; Geng, P.; Liu, H. J.; Teng, Y. Q.; Liu, Y. J.; Wang, Q. J.; Zhang, W.; Jin, L. T.; Jiang, L., Development of an electrochemical immunoassay for rapid detection of *E. coli* using anodic stripping voltammetry based on Cu@Au nanoparticles as antibody labels. *Biosensors & Bioelectronics* **2009**, 24 (7), 2155-2159.
 - (36) *Guidelines for drinking-water quality: First Addendum to Third Edition. Vol. 1, Recommendations*,. 3rd ed.; World Health Organization: Geneva, 2006.
 - (37) Matsushita, K.; Ameyama, M., D-glucose dehydrogenase from *Pseudomonas fluorescens*, membrane-bound. *Methods Enzymol.* **1982**, 89, 149-154.
 - (38) Ertl, P.; Unterladstaetter, U.; Bayer, K.; Mikkelsen, S. R., Ferricyanide reduction by *Escherichia coli*: Kinetics, mechanism, and application to the optimization of recombinant fermentations. *Anal. Chem.* **2000**, 72 (20), 4949-4956.
 - (39) Wang, Y. Y.; Claeys, L.; van der Ha, D.; Verstraete, W.; Boon, N., Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. *Appl. Microbiol. Biotechnol.* **2010**, 87 (1), 331-341.
 - (40) Cozier, G. E.; Salleh, R. A.; Anthony, C., Characterization of the membrane quinoprotein glucose dehydrogenase from *Escherichia coli* and characterization of a site-directed mutant in which histidine-262 has been changed to tyrosine. *Biochem. J* **1999**, 340, 639-647.

List of Figures

Figure 1. (A) Cyclic voltammograms of an etched alumina membrane-modified electrode in the presence of 1.0 mM ferrocenemethanol after each step of the biosensor construction procedure. Biosensor signal response is derived from the anodic peak currents. *E. coli* concentration = 10^6 cfu mL⁻¹. (B) Calibration plot of normalized biosensor signals derived from the voltammetric peak currents vs. logarithm of *E. coli* concentrations. Conditions: scan rate = 50 mV s⁻¹, antibody concentration = 0.1 mg mL⁻¹, pH 6.8.

Figure 2. Effect of antibody concentration on (A) loading amount of antibody, (B) biosensor signal response toward *E. coli*. Effect of pH on (C) loading amount of antibody, (D) biosensor signal response toward *E. coli*. Loading amount of antibody was measured by change in biosensor signal response after the antibody immobilization step. All biosensor signals are normalized against the signal response of the same biosensor in the absence of the immobilized antibody (I_{membrane}) for (A) and (C) or absence of *E. coli* cells ($I_{E.coli=0}$) for (B) and (D). Deviations of $I_{E.coli=0}/I_{\text{membrane}}$ and $I_{E.coli}/I_{E.coli=0}$ from 100% and 1 indicate antibody loading and presence of *E. coli* cells, respectively.

Figure 3. Specificity test to compare the biosensor signal response toward *E. coli* and two other bacteria species at four different concentrations (10^3 , 10^4 , 10^5 and 10^6 cfu mL⁻¹). Control measurements carried out in the absence of any bacteria species is added for reference (blank). Biosensor signal responses towards bacteria (I_{cell}) are normalized against the signal response of the same biosensor in the absence of bacteria ($I_{\text{cell}=0}$).

Figure 4. Linear sweep voltammetric responses of biosensors with captured (A) live, (B) VBNC and (C) dead *E. coli* cells towards successive addition of 10 and 80 mM glucose solution. (—) Binding with *E. coli*, (---) addition of 10mM and (····) 80 mM glucose solutions. (D) Normalized signal response of biosensors with captured (■) live and (◆) VBNC *E. coli* cells toward successive addition of glucose (10, 20, 40, 60 and 80 mM). Lines are nonlinear curve fits using Michaelis-Menten equation.

List of Schemes

Scheme 1. Nanoporous membrane-based biosensor design to illustrate the biosensor construction and *E. coli* cell analysis steps. Enlarged drawing shows the enzymatic oxidation of glucose in the presence of a redox probe shuttling between the cytoplasmic membrane of a captured *E. coli* cells and the biosensor.

Figure 1

Figure 2

Figure 3

Figure 4

Scheme 1