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A polyethylene glycol based microfluidic device integrated with nanoporous alumina membranes for simultaneous detection of multiple foodborne pathogens

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Abstract

Conventional biochemical methods for foodborne pathogen detection are time-consuming, labor-intensive and can only be used for single type bacteria detection with one sample. There is always an urgent need for fast, accurate and easy to handle devices for identification and monitoring of multiple foodborne pathogens at the same time. Herein, we present a non-biofouling polyethylene glycol (PEG) based microfluidic chip integrated with functionalized nanoporous alumina membrane for simultaneous electrochemical detection of two types of bacteria *E. coli O157:H7* and *Staphylococcus aureus* from the mixed samples. The experimental results demonstrated the specificity for target bacteria detection and the low cross-binding of non-target bacteria. The simultaneous detection of mixed bacteria sample of *E. coli O157:H7* and *Staphylococcus aureus* was also demonstrated. This sensor has a linear detection range from 10^2 CFU mL^{-1} to 10^5 CFU mL^{-1} with the limit of detection (LOD) around 10^2 CFU mL^{-1}.

Key words: Nanoporous alumina membrane; Electrochemical biosensor; Microfluidic chip; Foodborne pathogen

1 Introduction

Recent events of food contamination have triggered strong concerns of people for food safety. The food industry is having more and more stringent regulations for tolerable levels of food-borne pathogens. However, traditional techniques for food-borne pathogens detection such as Enzyme-Linked Immunosorbent Assay (ELISA) and culture and colony counting method are time and labor-intensive [1-4]. To solve these problems, indirect methods including electrochemical methods have been used for fast pathogen detection by monitoring the signal change from the transducer outputs [5-7]. There are numerous reports in the literature dealing with its application for rapid bacterial detection [8–10]. However, the traditional electrochemical devices have relative low sensitivities, which have hampered the wide application of this method. Moreover, traditional electrochemical methods often suffer from the false alarming caused by the uncertain specificity. So it is of much interest to develop a new electrochemical sensing system to overcome these obstacles for rapid detection of multiple bacteria contaminants in low concentration.

Nanoporous materials have been widely used in biosensing due to the high surface to volume ratio and enhanced sensitivity. Among them, nanoporous aluminum oxide membrane (AOM) is of particular interest due to its good biocompatibility as well as easy fabrication process [11-13]. Nanoporous alumina membrane has found wide applications such as ion channel detection [14], DNA hybridization sensing [15], virus detection [16], cell based biosensing [17] and bacteria detection [18-19]. However, the current nanoporous membrane based methods was only used for single type of

bacteria detection and could not realize the simultaneous detection of multiple types of bacteria from the mixed bacteria samples. The integration of suitable microfluidic chip with functionalized nanoporous alumina membranes is necessary to provide a multi-functional platform for simultaneous bacteria detection. Moreover, surface biofouling is always a challenge for traditional PDMS microfluidic devices. Recently, researchers started to use photocurable polyethylene glycol (PEG) polymer with low molecular weight to fabricate microfluidic devices due to its high resistance to swelling in aqueous environment [20-24]. Compared with PDMS based microfluidic device, PEG based microfluidic chip can significantly reduce sample loss, especially for low concentration detection.

In this paper, a polyethylene glycol (PEG) based microfluidic chip integrated with functionalized nanoporous alumina membranes was fabricated which enabled fast, sensitive and simultaneous electrochemical detection of two types of food-borne pathogens *E. coli O157:H7* and *Staphylococcus aureus* from the mixed samples. Two nanoporous alumina membranes were immobilized with anti-*E. coli O157:H7* antibody and anti-*Staphylococcus aureus* antibody, respectively. Then, the two functionalized membranes were integrated with PEG microfluidic device *via* UV-assisted bonding. The usage of PEG for microfluidic device fabrication could significantly reduce bacteria sample loss compared with PDMS device. The experimental results with pure bacteria samples showed specific responses and only a low degree of non-specific cross-binding. This chip was also tested for simultaneous detection of the specific bacteria in the mixed bacteria samples. It was demonstrated

that this chip could provide a fast and specific detection of *E. coli O157:H7* and *Staphylococcus aureus* at the same time with a linear detection range from 10^2 CFU mL^{-1} to 10^5 CFU mL^{-1} and a limit of detection (LOD) around 10^2 CFU mL^{-1}.

2. Materials and Methods

2.1 Functionalization of nanoporous alumina membrane

Nanoporous alumina membrane was purchased from Whatman, Inc., UK with 100 nm pore size. The membranes were 60 μm thick with 13 mm diameter. The membranes were firstly treated with 10% hydrogen peroxide and then washed with deionized water (DI water). The membranes were then boiled in deionized water (DI water) for 15 minutes and then immersed in silane-(PEG)5000-NHS (Nanocs Inc., USA) mixed with pure DMSO (Bio Basic Inc., Canada) at a concentration of 1%. The silane-(PEG)5000-NHS / DMSO solution was then reacted with the membranes for 2 hours at room temperature. Here, DMSO could minimize hydrolysis of the NHS groups and preserve the NHS group reactive. After 2 h, DI water and phosphate buffer solution (PBS, pH=8.3) was used to thoroughly wash the nanoporous alumina membrane, respectively. Finally, PBS solution with pH 9.0 was used to quench the remaining active NHS group to form carboxylate-terminated linear PEG chains on membrane surface to prevent non-specific adsorption. Then, 0.1 mg/ml anti-*E. coli O157:H7* or anti-*Staphylococcus aureus* antibody was added on the surface of nanoporous alumina membranes and incubated at 4 °C in a refrigerator for 24 h.

2.2 Fabrication of PEG based microfluidic device integrated with nanoporous alumina membrane

Fig. 1 shows the scheme for fabrication of PEG based microfluidic device integrated with functionalized nanoporous alumina membranes. Firstly, PDMS molds with designed pattern were fabricated using the soft lithography method. Then, PEG diacrylate (PEGDA, MW = 258, Aldrich) prepolymer solution with photo-initiator Irgacure 2959 was drop-dispensed on the PDMS molds. The assembly was put under an EXFO UV curing system (10 mw/cm^2) for partial photo-polymerization and then cooled down to room temperature. The PDMS molds were then gently peeled off to get PEG layers. The UV exposure time for each PEG layer was determined by the thickness. UV exposure time should be optimized to make the fresh polymer solution to form plates with desired degree of polymerization. So, the PEG plates were hard and easy to handle. At the same time, they could be covalently bonded together by complete UV polymerization. Then, the three incompletely polymerized plates and functionalized nanoporous alumina membranes were assembled and bonded together via UV assisted bonding with PEGDA precursor. PEGDA precursor solution was added on the contact area of nanoporous membrane and PEG layers. The hydrogel precursor solution was able to infiltrate into the nanopores and then was crosslinked to anchor the nanoporous membrane with PEG layers. All the parts were covalently bonded together.

2.3 Sensing mechanism

The principle of simultaneous detection for two types of bacteria of the fabricated PEG microfluidic devices integrated with nanoporous membranes was shown in Fig. 2. The whole device is separated by functionalized nanoporous alumina membranes with upper and bottom compartments. The membranes on bottom chamber 1 and bottom chamber 2 are modified by anti-*E. coli O157:H7* and anti-*Staphylococcus aureus*, respectively. Each chamber underneath the nanoporous membrane works as an independent sensing chamber with a Pt working electrode, while the upper chamber works has a common Pt reference electrode. As a result, each bottom chamber could work independently with its own planar working electrode and the common reference electrode without influencing the other bottom sensing chamber. For example, when nanoporous membrane on bottom chamber (1) captures specific bacteria type 1, the impedance amplitude between common upper chamber and bottom chamber (1) will increase due to the blocking of electrolyte current. The "open' or "close" status of nanoporous membrane of bottom chamber (2) will not affect the impedance signals in bottom chamber (1). Both bottom chambers could work independently with the independent working electrodes.

2.4 Impedance single measurement for bacteria detection

Two relays were connected with the two platinum microelectrodes in bottom chambers with the common terminal connected with the impedance analyzer. For the impedance measurement, pure PBS was first injected into bottom chambers. Samples

of mixed *E. coli O157:H7* and *Staphylococcus aureus* with different concentrations were then injected into the common upper chamber. To testify the specificity of this simultaneous detection system, samples of only *E. coli O157:H7* and only *Staphylococcus aureus* with various concentrations were also used for testing. The measurement of impedance was conducted by the electrochemical analyzer VersaSTAT3 (METEK) with 50mVpp voltage within frequency range from 1 Hz to 10 kHz at room temperature. The software V3-Studio was used to record the impedance variation (electrochemical signal). The normalized impedance change (NIC) was used to represent impedance amplitude change relative to control data. NIC is expressed by the following equation:

$$NIC = \frac{Z_{sample} - Z_{control}}{Z_{control}} \times 100$$

2.5 Fluorescence imaging

The target bacteria captured on the complimentary antibody modified membrane were confirmed by sandwich type immunoassay using fluorescence dye labelled antibody. After the bacteria cells were captured by specific antibodies on the functionalized nanoporous alumina membrane, 0.5 mg/mL fluorescein (FITC) conjugated anti-*E.coli O157:H7* and rhodamine (TRITC) conjugated anti-*Staphylococcus aureus* antibodies were used to form a sandwich structure. After incubation at room temperature for 30 minutes, the nanoporousmembrane was rinsed by phosphate-buffered saline (PBS) solution (0.01M, PH=7.4) for three times to remove physically adsorbed fluorescence antibodies from the membrane surface. A

fluorescence stereo microscope was used to take fluorescence images of nanoporous alumina membranes.

3. Results and discussion

3.1 Fabrication of microfluidic chip

A PEG based microfluidic chip integrated with two nanoporous alumina membranes was successfully fabricated for simultaneous detection of two types of foodborne pathogens *E. coli O157:H7* and *Staphylococcus aureus*. PEGDA (MW=258) was chosen for microfluidic device fabrication which was demonstrated to have good swelling resistance and mechanical property. Fig. 3 shows the image of the fabricated PEG chip with two integrated nanoporous alumina membranes. During the PEG device fabrication, the UV exposure time was optimized to ensure that there were enough active acrylate groups left on partially polymerized PEG layer for following bonding step. Fig. 4a shows the SEM image of the PEG channel formed by bonding between two PEG layers. The two PEG layers were completely sealed where the interface could not be observed. Fig. 4b shows the SEM image of functionalized nanoporous alumina membrane. For the bonding between nanoporous alumina membrane and PEG layer, PEGDA pre-polymer solution was added to the contact area between the membrane and the PEG layer to infiltrate into the nanopores. After UV curing, nanoporous alumina membranes and PEG layers were firmly bonded together. Fig. 4c shows the SEM image of bonding boundary of nanoporous alumina membrane and PEG layer.

3.2 Anti-biofouling testing

To evaluate the non-biofouling feature of PEG to prevent bacteria adhesion, labeled *E. coli O157:H7* and TRITC labeled *Staphylococcus aureus* with concentration of 10^6 CFU/mL were added into PEG/PDMS micro-channels and bare nanoporous alumina membrane/PEG grafted nanoporous alumina membrane for comparison. To visualize the bacteria adsorption, the bacteria cells were added on the samples for 4 hours and then followed by gentle washing with PBS solution. Fig. 5a shows the representative fluorescence image of *E. coli O157:H7* adsorption on PDMS and PEG channels, respectively. It was shown that bacteria adhesion on PEG surface was much lower than that on PDMS surface. There were few bacteria cells adhered on non-biofouling PEG surface. The quantitative analysis showed that *E. coli O157:H7* adhesion on PEG surface was around 17% with respect to that on PDMS surface, and *Staphylococcus aureus* adhesion on PEG surface was around 11% with respect to that on PDMS surface. This demonstrated that PEG based microfluidic device could significantly prevent bio-fouling and reduce sample loss. Fig. 5b shows the representative fluorescence image of *Staphylococcus aureus* adhesion on bare nanoporous alumina membrane and inert silane-PEG layer coated nanoporous alumina membrane. There were few bacteria cells adhered on silane-PEG layer coated membrane. The quantitative analysis showed that *E. coli O157:H7* and *Staphylococcus aureus* adhesion on silane-PEG layer coated membrane was reduced to 14% and 8% with respect to bare membrane, respectively. It demonstrated that the formation of PEG layer on membrane could significantly reduce non-specific bacteria

adhesion.

3.3 Bacteria capture on functionalized nanoporous membrane

When bacteria sample was injected into the biochip, specific antibody immobilized nanoporous alumina membrane could capture the complimentary bacteria cells. Fig. 6a shows the SEM images of *E. coli O157:H7* and *Staphylococcus aureus* captured on specific antibody immobilized nanoporous alumina membrane, respectively. The rod-shaped *E. coli O157:H7* and round-shaped *Staphylococcus aureus* were successfully captured on the nanoporous membrane surface. To testify the specificity of this device for *E. coli O157:H7* and *Staphylococcus aureus* detection, samples of only *E. coli O157:H7*, only *Staphylococcus aureus* and mixture of the two bacteria (ratio 1:1) with concentration of 1.0×10^6 CFU/mL were injected into the chip for simultaneous detection. Sandwich type immunoassay using FITC-labeled anti- *E. coli O157:H7* antibody and TRITC-labeled anti-*Staphylococcus aureus* antibodies were used for bacteria labeling. As shown in Fig. 6b, for only *E. coli O157:H7* sample detection, *E. coli O157:H7* detection unit showed relative strong green signals and *Staphylococcus aureus* detection unit showed very weak green signals. This demonstrated that the nonspecific binding of *E. coli O157:H7* on *Staphylococcus aureus* antibody immobilized nanoporous alumina membrane was low. The similar result was observed for only *Staphylococcus aureus* sample, where *Staphylococcus aureus* detection unit had the relative strong red signals and *E. coli O157:H7* detection unit has the weak red signals. For the mixture of the two bacteria samples,

both detection units showed strong green and red signals respectively, which demonstrated the simultaneous detection for *E. coli O157:H7* and *Staphylococcus aureus*. Fig. 6c shows the high resolution fluorescence images for rod-shaped *E. coli O157:H7* and round shaped *Staphylococcus aureus* captured on nanoporous alumina membrane.

3.4 Impedance sensing

To testify the functionality of this device for multiple bacteria detection, *E. coli O157:H7* sample, *Staphylococcus aureus* sample and mixture of the two bacteria (ratio 1:1) with various concentrations were tested. The samples were injected into the chip and incubated for around 30 minutes and followed by PBS washing. Fig. 7a shows the normalized impedance change (NIC%) of the chip with two detection units for only E. coli O157:H7 sample detection at 100 Hz with different concentrations from 10^2 to 10^5 CFU/mL. PBS solution is used as the control. The NIC amplitude is 10.3±2.6 % for 10^2 CFU/mL, 19.8±4.1% for 10^3 CFU/mL, 38.5±8.8% for 10^4 CFU/mL, and 61.6±11.7% for 10^5 CFU/mL in *E. coli O157:H7* detection unit. There is no significant signal increase for only *E. coli O157:H7* samples in *Staphylococcus aureus* detection unit, which was around 4% ~5% change due to nonspecific binding. The similar result was found for only Staphylococcus aureus sample in Fig. 7b. The NIC amplitude is 18.5±4.6% for 10^2 CFU/mL, 24.6±5.2% for 10^3 CFU/mL, 42.7±9.8% for 10^4 CFU/mL and 68.7±13.1% for 10^5 CFU/mL for *Staphylococcus aureus* detection unit. The NIC amplitude is in the range 3% to 6% for *E. coli O157:H7*

detection unit due to the non-specific binding. Fig. 7c shows the NIC amplitude change for the mixture of the two bacteria. NIC amplitude is 14.4±3.9% for 10^2 CFU/mL, 23.5±5.4% for 10^3 CFU/mL, 46.5±10.2% for 10^4 CFU/mL, 67.4±14.2% for 10^5 CFU/mL for E. coli O157:H7 detection unit. NIC amplitude is 22.7±5.7%, for 10^2 CFU/mL 30.4±6.4% for 10^3 CFU/mL, 51.7±11.9% for 10^4 CFU/mL, 77.5±14.7% for 10^5 CFU/mL for *Staphylococcus aureus* detection unit.

The impedance change of nanoporous alumina membrane detection unit is due the blocking of nanopores by the complimentary bacteria capturing on the membrane surface. As shown in Fig. 7a and Fig. 7b, both detection membrane units showed good specificity for the target bacteria detection with the detection limit of 10^2 CFU/mL. The impedance signal change caused by non-target bacteria binding was around 5% which was much lower than the change caused by target bacteria capturing in the range from 10^2 to 10^5 CFU/mL. The impedance change under 10^2 CFU/mL could not be distinguished from the non-specific bacteria binding. As shown in Fig. 7c, the mixture of the second type bacteria did not interfere the impedance signal much compared with the signal with single type bacteria in Fig. 7a and Fig. 7b. The linear relationship NIC%=18.216 log(CFU)-7.6 with R^2=0.972 is found for *E. coli O157:H7* detection and NIC%=18.585 log(CFU)-0.885 with R^2=0.9527 for *Staphylococcus aureus* detection in the range from 10^2 to 10^5 CFU/mL.

4. Conclusion

In summary, we have designed a novel PEG based microfluidic device integrated with functionalized nanoporous alumina membranes for multiple foodborne pathogen detection. The usage of PEG for microfluidic device fabrication and the grafting PEG monolayer on nanoporous alumina membrane can significantly reduce non-specific bacteria binding. This device has successfully demonstrated the simultaneous electrical impedance detection of specific bacterial strains from the mixed *E. coli O157:H7* and *Staphylococcus aureus* samples without labeling. Tests with pure and mixed bacteria samples demonstrated that the device could perform sensitive and specific detection for the above two types of bacteria. The present work promises a simple and sensitive platform for detection of multiple foodborne pathogens at the same time for food safety and environmental monitoring.

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Figure Captions

Figure 1 Schematic diagram of fabrication procdures for PEG microfluidic device integrated with functionalized nanoporous alumina membranes for multiple food pathogen detection.

Figure 2 The principle of simultaneous detection for two types of bacteria using the microfluidic device integrated with nanoporous membranes.

Figure 3 Image of fabricated PEG microfluidic chip integrated with nanoporous alumina membranes.

Figure 4 (a) SEM image of the PEG channel formed by bonding between two PEG layers; (b) SEM image of functionalized nanoporous alumina membrane; (c) SEM image of bonding boundary of nanoporous alumina membrane and PEG layer

Figure 5 (a) Representative fluorescence images of *E. coli O157:H7* adsorption on PDMS and PEG surfaces and quantitative data for *E. coli O157:H7 and Staphylococcus aureus* adsorption on PDMS and PEG surfaces; (b) Representative fluorescence image of *Staphylococcus aureus* on bare nanoporous alumina membrane and silane-PEG layer grafted nanoporous alumina membrane and quantitative data for *E. coli O157:H7* and *Staphylococcus aureus* adsorption on bare and silane-PEG layer grafted nanoporous alumina membrane.

Figure 6 (a) SEM images of *E. coli O157:H7* and *Staphylococcus aureus* captured on specific antibody immobilized nanoporous alumina membranes; (b) Fluorescent

images of nanoporous membrane detection units for samples of only *E. coli O157:H7* sample, only *Staphylococcus aureus* and mixture of the two bacteria samples; (c) Fluorescence images for rod-shaped *E. coli O157:H7* and round-shaped *Staphylococcus aureus* captured on nanoporous alumina membrane.

Figure 7 Impedance sensing for simultaneous detection of *E. coli O157:H7* and *Staphylococcus aureus* for (a) only *E. coli O157:H7* sample, (b) only *Staphylococcus aureus* sample, (c) mixed bacteria sample with ratio 1:1.