Ultra-Sensitive Electrochemical Detection of *E. coli* Using Nano-Porous Alumina Membrane

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ABSTRACT

A combinational method has been developed for ultra-sensitive detection of *E. coli*. The method combined biological, nano-fabrication and electrochemical techniques to achieve highly specific and ultra-sensitive detection of bacterial pathogen. *E. coli* K12 was used as the target bio-agent to demonstrate the detection model. Nano-porous alumina membrane was chosen as filtration substrate to separate *E. coli* from the test samples because of their desirable physical properties. The surface of the membrane was blocked and not to bind to protein molecules. Bacterophage M13 was genetically modified with a histag on its minor coat protein III. This phage was used to infect its specific bacterial host *E. coli* K12 and replicated inside of the host. The nano-porous alumina membrane was used to filter exceeded phages and separate infected *E.coli* from the test sample. The replication time of bacterophage M13 was investigated. After a short period of culture, the replicated bacterophage was released from the host cell. The phage was increased 10^2 -10^5 fold in 2 -4 hrs. These baterophage were captured by anti-histag antibody immobilized on the detection electrode and detected electrochemically. Various surface modification approaches on the detection electrode were tested to improve the efficiency of target recognition and detection sensitivity. This combinational method can be applied to the detection of various pathogenic bacteria.

Key words: nano-porous alumina membrane, *E. coli*, phage, electrochemical, detection

1 INTRODUCTION

Food borne illnesses contribute to the majority of infections caused by pathogenic microorganisms. Toxin producing *E. coli* can cause stomach illness, bloody diarrhea, dehydration or even death in serious cases. It can contaminate ground beef if fecal matter or other contaminated meat is mixed during the meat slaughter or packing operations. Among these pathogenic *E. coli*, *E. coli* O157:H7 is firmly associated with hemorrhagic colitis [1]. As a result of this association, *E. coli* O157: H7 was designated as an enterohemorrhagic *E. coli*. Based on a 1999 estimate, 73,000 infections and 61 deaths occur in the United States each year [2]. In year 2007, several recalls of *E.coli* O157:H7 contaminated ground beef involved three companies and over 20 million pounds products which resulted in huge economic loss. Current tests for the detection of pathogenic *E. coli* depend on the time consuming culture method. PCR amplification is another commonly used method. However, it cannot identify alive or died bacteria and lacks accuracy due to contamination problems. Reliable and accurate molecular biology methods are time consuming and have resulted in delayed recalls of contaminated meat products. Developing rapid and accurate detection technologies will prevent news headlines like, "The Department of Agriculture and Cargill Meat Solutions Corp. today recalled more than 1 million pounds of ground beef products because they may be contaminated with *E. coli* O157:H7 bacteria. The recall comes just weeks after one of the largest meat recalls in history -- more than 20 million pounds. The recall by the Topps Meat Co. sent the New Jersey firm out of business". It is clear that rapid and accurate detection of contaminated food will save lives and prevent economic disasters in agriculture. Detection of food borne pathogens has led to increased research interest. New technologies in the biosensor development are improving the sensor characteristics such as sensitivity, reliability, simplicity and economic viability [3,4,5]. Here we report a combinational method for ultra-sensitive detection of *E. coli* K12. This detection method can be applied to other pathogenic bacteria including *E. coli* o157:H7.

2 METHODS

2.1 Electrochemical Detection

The detection system contains three parts: target recognition, signal amplification and electrochemical detection. Bacterophage was used for both target

recognition and signal amplification through its natural features (see electrochemical detection model in Figure 1.).

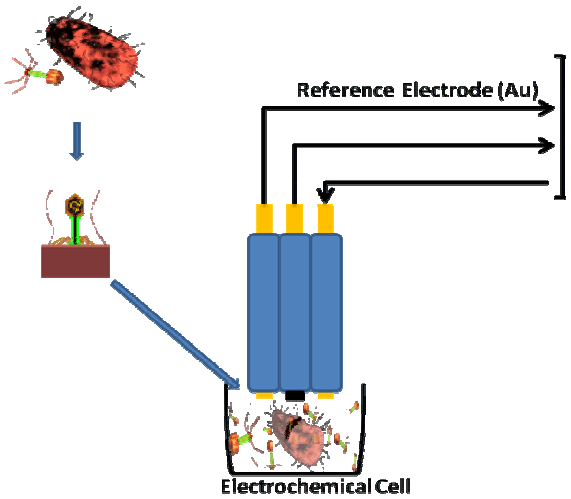

Figure 1. An overview of the detection mechanism Bacterophages infect their host bacteria specifically and are replicated in the host cell. The host bacteria release the replicated phages into culture medium. These bacterophages are captured by their specific antibody on the detection electrode and detected electrochemically.

2.2 Genetic Modification of Phage M13

A peptide phage display cloning vector from New England Biolab was used to insert a 60 base pairs double strand oligonucleotide which codes a poly-histidin peptide at the N-terminal of coat protein gene III. The sequence of the oligonucleotide is: 5'-CATGCCCGGGTACCTTTCTATTCTCACTCTCA TCATCATCATCATCACTCGGCCGAAACATG-3'. Two single strand oligonucleotides corresponding to a partial sequence of the top and bottom strands with 15 base pairs overlap were designed and synthesized. The sequence of the top strand is: 5'-GTA CCT TTC TAT TCT CAC TCT CAT CAT CAT CAT CAT CAC TC -3'. The sequence of the bottom strand is: 5'- GGC CGA GTG ATG ATG ATG ATG ATG AGA GTG AGA ATA GAA AG -3'. Annealing and primer extension were performed to obtain a double strand oligonucleotide with a completed a sequence which was inserted into vector between restriction sites Acc65 I and Eag I. General molecular cloning approaches were used to transform ligation sample into *E. coli* K12. Plagues were isolated from the agar plate and re-suspended in PBS. This phage sample was used to infect host bacteria for its mass production.

2.3 Characterization of Histaged Phages M13

To characterize genetically modified bacterophage M13, nickel-HRP conjugate was used to detect poly-histidin on phage surface. 0.5 ul genetically modified and unmodified M13 phage samples were spotted on nitrocellulose membrane. 0.5 ul of histaged GFP was used as positive control. After blocking, the membrane was incubated with nickel-HRP conjugate. Chemiluminescence detection was used to confirm the presence of histag.

2.4 Phage infection and generation time studies

Over night culture of *E. coli* K12 was diluted in LB broth at 1: 100, 1:10,000, 1:100,000 ratios. Spectrum OD600 reading was taken to estimate bacterial density. Each diluted samples was plated on LB agar plates to determine the number of bacteria in the culture. Bacterophage M13 infection was carried out by incubating bacterial samples with histaged phage M13 at room temperature for 40 minutes. The exceeded phages were removed by using 0.2 nm spin filter. Infected *E. coli* K12 were then recovered and cultured in LB medium. To determine phage replication time course, the samples from 2, 4.5 and 6 hrs cultures were plated on *E. coli* K12 top agar plates and incubate at 37^{o}C for over night. Plagues were counted from over night cultures.

2.5 Surface modification of detection electrode

Immobilization of antibody on the detection electrode was tested on gold wires. Sonication was used to clean the gold surface before modifications. Cleaned gold wires were soaked in a PBS solution containing 10 µg/ml mouse IgG or anti-biotin antibody at room temperature for 60 minutes. Goat anti-mouse IgG and alkaline phosphatae (AP) conjugate or biotin labeled AP was used to confirm mouse IgG immobilized on the gold wires. Chemiluminescence detection was recorded by CCD imaging. After confirmation, the same approach of surface modification was used to immobilize a monoclonal anti-histag antibody on the gold detection electrodes.

2.6 Electrochemical detection of bacterophage M13

A pure *E. coli* K12 culture was diluted to100 bacteria/ml. One mili-liter this diluted sample was filtrated through nano-porous membrane with the hole size of 200 nm. Bacterophage M13 infection and replication was carried out as described above. The antibody modified detection electrode incubated with the phage samples replicated from infected *E. coli* K12. Electrochemical signals generated from the interaction of histaged bacterophage M13 and anti-histag antibody were detected.

3 RESULTS AND DISCUSSION

3.1 Genetic Modification of Bacterophage M13

A short double strand oligonucleotide was inserted into a phage display vector M13KE (Fig. 2 left) at the cloning site of between Acc65 I and Eag I. The inserted sequence of this oligonucleotide is : **5'-**GGTACCTTTCTATTCTCACTCT**CATCATCATC ATCATCAC**TCGGCCG-3'. The bold sequence codes a polyhistidin peptide, and the underline sequences are restriction sites Acc65 I and Eag I. This vector was transformed and replicated in *E. coli* K12. Genetically modified phage M13 was confirmed by chemiluminescence test. Fig. 2 (right) showed that histag coat protein III in M13 was detected by Nickel-HRP conjugates. Unmodified phage M13 did not generate a light signal.

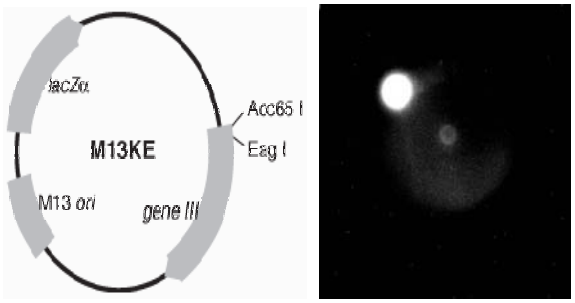

Figure 2. Characterization of genetically modified bacterophage M13 The map of phage displry vector is on the left. Chemiluminescence detection of histaged M13 is showed on the right. Top left is positive control of histaged GFP. Top right is negative control of unmodified M13. Bottom center is modified phage M13.

3.2 Investigation of Bacterophage M13 Generation Time

Results from the study of M13 generation time were show in Figure 3. As less as 7-10 infected *E. coli* K12 bacteria produced 200, 3,600 and 667,000 new phages in 2, 4.5 and 6 hrs respectively. Bacteria concentration was determined on agar LB culture plates. The result showed that in 2, 4.5 and 6 hrs time period, the replication times are 70, 518 and 95,300 fold. This result has been repeated several times.

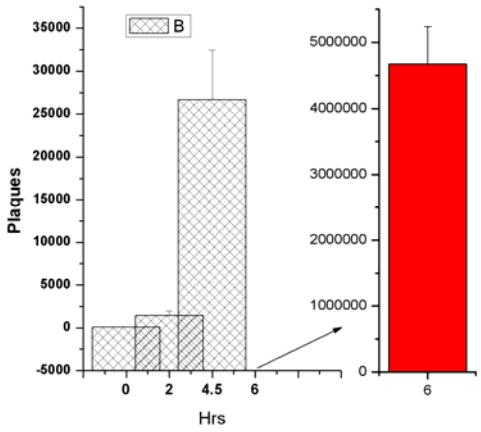

Figure 3. Bacterophage M13 generation time Phage M13 replicated in 7-10 infected *E. coli* K12 cells. The numbers of replicated phages are showed in: 0 hr, 2 hrs, 4.5 hrs and 6 hrs (red).

3.3 Antibody Immobilization

Antibody immobilization on gold wire was confirmed by chemiluminescence assay. Fig.4 showed that mouse IgG was detected by goat anti-mouse alkaline phosphatase (AP) conjugates, and anti-biotin antibody was detected by biotin labeled AP. In both cases, unmodified gold wire did not produce chemiluminescence signal.

Figure 4 Chemiluminescence detection of antibodies immobilized on gold wire, left: mouse IgG on gold wire, right: anti-biotin antibody on gold wire.

3.4 Detection of *E. coli* K12

Detection of *E. coli* K12 was demonstrated through the processes of bacterophage M13 infection, replication and detection. Alumina nano-porous membrane showed a great advantage over other commercial membranes. Bacteria stuck on the filtration membrane are common problems when using nitrocellulose or PVDF membrane. That reduces biological activity of the cells and affects phage replication in the host. In contrast, alumina materials are not sticky to biological samples. It is easier to re-suspend filtered bacteria into culture medium.

A set of micro-electrodes were fabricated on a glass wafer for electrochemical detection. Fig. 5 is an image of the detection device. The results of electrochemical detection of histaged bacterophage M 13 were showed in Figure 6.

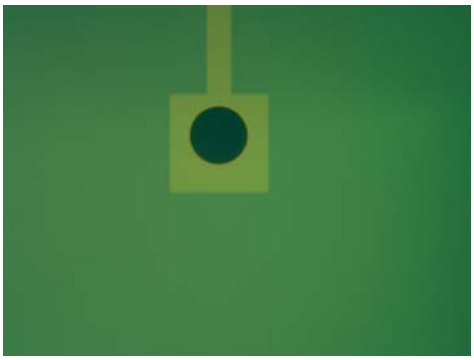

Figure 5. Microscopic images of the 300 micron gold detection (black circle).

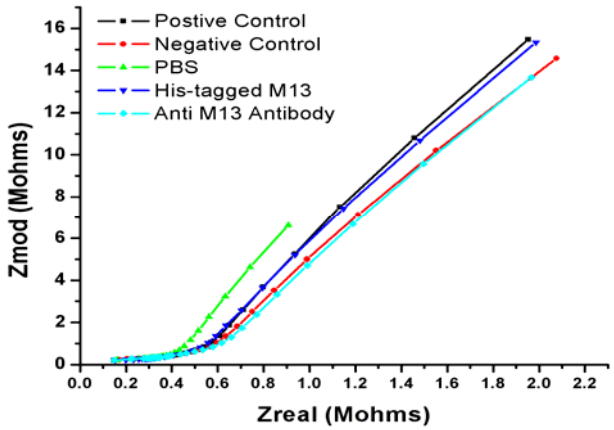

Figure 6. Electrochemical nyquist plot of bacterophage M13 detection on 300 micron antibody coated electrode. The spectra clearly shows the specific detection of M13 phage at AC 100 mV rms.

4. CONCLUSION

The advances in biotechnology, bio-molecular electronics, nano-technology and other sciences have led to the spawning of new and rapid methods of food pathogen detection that have the potential for on-site testing and thereby revolutionizing food product quality control. The advantages of using phage electrochemical detection method are: quicker, sensitive, easy to process and simple devices. This detection model can be applied to the detection of other pathogenic bacterial contamination in food, e g. detection of *E. coli* O157:H7 by its specific bacterophage KH1, and detection *Salmonella* by its specific bacterophage P22.

5. ACKNOWLEDGEMENTS

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