A net fishing enrichment strategy for colorimetric detection of *E. coli* O157:H7

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Abstract

The strict regulatory requirements for pathogen monitoring in food systems to ensure safety demands that the detection method can recognize small numbers of pathogens. Although significant efforts on the development of biosensors have been reported with marked improvement in sensitivity, appropriate enrichment strategies are still needed in a detection scheme. Herein we proposed a unique strategy for the enrichment of pathogens, based on a concept termed as net fishing, to capture target bacteria with antibodies functionalized on porous substrates to fish the target from samples. Because of their large surface area porous nitrocellulose membranes (NC membranes) were used as substrates for target capture. By immersing the antibody modified NC membrane into liquid samples and upon mixing, we expect to anchor target pathogens on the membrane due to the specific interaction between antibody and target, where the nonspecific enrichment could be reduced compared to methods such as filtration. Meanwhile the net fishing enrichment requires 2 hours, less than common culture methods. An enzyme enhanced procedure was performed with the NC membrane after the capture to obtain colorimetric signal. Gold nanoparticle (GNP) probes conjugated with horseradish peroxidase (HRP) and antibody were used to label capture target bacteria, and the presence of pathogens was determined by the colorimetric signal generated from HRP catalyzed TMB reaction. With *E. coli*

O157:H7 as target, we investigated the detection performance of the proposed net fishing strategy with an enzyme amplification step for colorimetric reporting. After optimization of detection conditions, we show that as low as 100 CFU/ml *E. coli* O157:H7 could be detected. The feasibility was then demonstrated using pineapple juice as a food model.

Keywords

Pathogen enrichment, nitrocellulose membrane, colorimetric detection, biosensors, *E. coli* O157:H7.

Introduction

Pathogenic microorganisms in food could cause serious problems to public health, food industry and environment, thus it is not surprising that strict regulatory requirements are imposed to secure the safety of food. Until now, biosensors based on various analytical techniques and strategies have been reported with improved detection sensitivity.[1-8] However, due to the limited detection capability in large volume samples, there is still a big gap between the reported sensitivity and the regulatory requirement. To bridge the gap, steps to enrich bacteria from samples is usually performed using methods such as culturing, filtration and concentration with NPs. Furthermore, selective enrichment of a particular bacteria is more valuable and attractive compared to increasing the number of all of the pathogens in the sample to minimize the effect of non-target signal. Culturing is one of the most common methods that can exponentially increase the amount of bacteria from the tested

samples. However to increase the number of pathogens culturing is performed, which usually requires 8-48 hrs, limiting its practical application.[9-12] Unless pre-culturing and selection of target bacteria is performed followed by another round of culturing, the number of target as well as non-target bacteria will be increased and may influence the detection of the target due to non-specific signals. Filtration should be the simplest method to increase the amount of bacteria in the samples,[13-15] and the concept can be extended to process large volume of samples in a short time.[16, 17] However, fractions in matrixes larger than the size of bacteria would be left in the concentrated samples to influence the determination. Besides, depending on the size rather than the type of pathogen, filtration cannot separate the target bacteria from the mixture of bacteria in samples. Benefiting from the development of nanotechnology, magnetic nanostructures modified with antibody or other recognition ligands were introduced to concentrate bacteria, enabling the selection of target bacteria.[18-21] However, the nonspecific binding of other materials in the sample onto the magnetic nanoparticles and the sticky nature of the common food matrixes would influence enrichment. Assisted by a proper enrichment method to increase the number of target bacteria before detection, better selectivity could be obtained and more specific onsite sensors could be developed.

The focus of this work is on *E. coli* O157:H7, a pathogen known to induce serial illnesses from mild watery diarrhea to life-threatening hemorrhagic colitis with symptoms of bloody diarrhea and thrombotic thrombocytopenic purpura,[22, 23] with possible mortality rates as high as 40%.[24] As low as 100 cells of *E. coli*

O157:H7 could cause the pathogen to grow in the intestine of susceptible patients and produce shiga toxins.[25, 26] In the last decade, numerous outbreaks of contaminated food implicating *E. coli* O157:H7 in juice and other foods have occurred. Several analytical techniques for the detection of *E. coli* O157:H7 have been developed.[27-34] Of the reported methods, enzyme amplification has been found to be an attractive strategy to improve the signal by an order of magnitude due to enzyme-catalyzed reaction, while the catalyzed product of suitable substrate could denote an obvious change in color enabling recognition by naked eyes. Colorimetric approach is an attractive method which could utilize an enzyme to amplify the signal of the target pathogen. However, a concern is that the activity of the enzyme might be influenced by the complexity of food matrix which requires pretreatment to enrich targets from the samples.

NC membrane due to its porous structure provides a large surface area. After modification with antibody, we expect the large surface area to assist in the capture of the target. Herein with the antibody modified NC membrane, we propose a unique strategy, termed as net fishing, to enrich and separate target bacteria from samples. The porous structure of antibody modified NC membranes were used as nets to fish the target bacteria in liquid samples. Unlike the filtration strategy, the target bacteria could be captured and enriched based on the specific interaction between antibodies and the corresponding bacteria, thus the nonspecific enrichment of non-target bacteria would be reduced. By interacting the modified NC membrane with the liquid samples under intensive mixing, an improved capture of target bacteria could be achieved.

Compared with the culture based enrichment routes, the proposed net fishing approach does not need long culture time with less influence from the proliferation of non-target bacteria due to the specificity of antibody. The captured target pathogens on NC membrane were determined with an enzyme amplified colorimetric method. Gold nanoparticles (GNPs) modified with antibody and HRP were used as probes to further improve the signal to detect the target. *E. coli* O157:H7 was chosen as the model organism to investigate the sensor performance based on net fishing enrichment and HRP amplification. The pathogen was heat killed to avoid the influence of proliferation. With a short time enrichment, excellent sensitivity was demonstrated. With a sandwich-like pattern comprising of antibody modified on the nitrocellulose membrane-target bacteria-antibody and HRP modified GNP probes, as low as 100 CFU/ml of *E. coli* O157:H7 could be recognized with the generated colorimetric signal, exhibiting an improved sensitive compared to the methods which use similar sandwich-like pattern such as the conventional ELISA [35, 36], nanoparticle enhanced ELISA [3] and magnetic enrichment-assisted ELISA methods [37, 38]. Validity of the method to detect pathogens in pineapple juice showed the feasibility of the proposed method in real food samples.

Experimental section

Chemicals and agents

Sodium Citrate, HAuCl₄ and Na₂CO₃ were purchased from Sigma (St. Louis, MO). Streptavidin Poly-HRP and sulfo-NHS-LC-biotin was obtained from Thermal Scientific (Rockford, IL). Tetramethyl benzidine (TMB) was bought from Moss, Inc.

(Pasadena, MD). NC membrane was purchase from mdi Membrane Techonologies INC (Harrisburg, PA) Polyclonal antibody (01-95-90) from goat against *E. coli* O157:H7 and *E. coli* O157:H7 were obtained from KPL (Gaithersburg, MD). All of the materials were used as received without further purification.

Preparation of GNP probes.

Before use, all glassware in experiments was cleaned in a bath of 3:1 HCl: HNO₃ solution and rinsed with Milli-Q system purified water. GNPs for the probes were synthesized following the reported method.[39] Briefly, 1 ml of 1% HAuCl₄ was added to 100 ml of water and heated to boiling with strong stirring. Then 1 ml of 1% sodium citrate was quickly added to the boiling solution. The obtained mixture was kept in boiling state for 10 minutes and cooled in room temperature and kept at 4°C until modification with antibody.

The modification of GNPs with antibody against *E. coli* O157:H7 and biotinylation was performed based on our previous work.[6, 8] 1 ml of GNPs was mixed with 1 µl of 0.5 M Na₂CO₃ and 100 µl of 10 mM phosphate buffer (pH 7.4), then 10 µg antibody was injected, followed by gentle shaking for 2 hours. The obtained antibody modified GNPs solution was added to 122 µl of 5% (w/v) casein and shaken overnight. The modified GNPs was then centrifuged at 10,000 rpm for 10 minutes to remove unbound molecules, washed and redispersed in 1 ml of 10 mM phosphate buffer. 10 µg of sulfo-NHS-LC-biotin was added to the product and shaken for 1 hour for biotinylation. 100 µl of 5% (w/v) casein was injected into the obtained solution and shaken for 1 hour to block the residual surface of GNP probe. The

prepared GNP probes were washed and redispersed in 100 μ l of 0.5% (w/v) casein in 10 mM PBS and kept at 4°C until use.

Detection of *E. coli* O157:H7

The NC membrane was shaped as strips with 4 mm $\times$ 25 mm dimension. 28 μ l of 33 μ g/ml antibody in 10 mM PBS with 5 ppm casein (wt%) was dropped onto the NC membrane strip and the strip was dried at 37°C for 2.5 hours. The strips were then immersed in 10 ml samples of 10 mM PBS in various concentration of *E. coli* O157:H7 along with 2.5 μ l GNP probes. The samples with strips was gently shaken for 2 hrs to allow the sample passing through the porous NC membrane to capture *E. coli* O157:H7. The NC membrane was washed 3 times with PBS and immersed in 1 ml of PBS, followed by addition of 5 μ l of 10 μ g/ml streptavidin Poly-HRP and gently shaken for 5 min. After washing 6 times with PBS, the strip was immersed in 0.11% (v/v) TMB in PBS and gently shaken for 10 min. The UV-vis spectra of the resulting solution were recorded. The normalized absorption (absorption at 661 nm subtracting absorption at 535 nm) was used to determine the concentration of *E. coli* O157:H7. All of the detections were performed in triplicate.

Pineapple juice was used as a model food system to demonstrate the feasibility of the proposed method. The pH of the pineapple was adjusted to 7.4 with NaOH before detection. To reduce the influence of thickening agents in pineapple juice, the juice was diluted with PBS in 1:1 ratio. Inoculated with a certain amount of *E. coli* O157:H7, the pH-adjusted pineapple juice was tested with the net fishing method.

Characterization

UV-vis spectra were collected with a Jasco V570 UV/Visible/NIR spectrophotometer (Jasco, Inc., Easton, MD). Transmission electron microscopy (TEM) images of GNP probes were recorded with a FEI Tecnai G2 20 transmission electron microscope operated at 100 kv. Scanning electron microscopy (SEM) images were collected with a FEI NOVA nanoSEM (FEI Co., Hillsboro, OR) operated at 5.0 kV.

Results and discussion

NC membrane is widely used in bio-analysis because it can be easily modified with antibody. Its porous structure and capillary property enables the application of NC membrane in the lateral flow detection. Besides, the porous structure provides a large surface area benefiting its interaction with NC membrane as detection substrate. Based on the structure and surface chemical feature, we used NC membrane as the substrate for the proposed net fishing concept to separate and enrich target bacteria from liquid samples. The inset SEM image in Scheme 1 illustrates the typical morphology of the NC membrane used. It can be seen that most of the pores are larger than 5 μm , suggesting that there will be very limited bacteria in the samples, target or non-target, anchored on the NC membranes due to the size. Instead, for selective capture of the target bacteria, antibody was conjugated onto the NC membrane. The whole procedure of conjugation and detection is shown in Scheme 1. First, the NC membrane was modified with antibody, not only on the surface but also in the pores of the membrane, enabling the capture of bacteria which occurred at the whole modified membrane. Because it is known that NC membrane could easily bind

protein while the large surface may induce some nonspecific interaction, therefore casein was used in the antibody solution as well to block the surface of NC membrane. Meanwhile it was also noticed that the GNP probes would be directly attached to the NC membrane. As shown in Figure S1, it can be observed that the casein block could reduce the nonspecifically bound GNP probes on the NC membrane. After casein blocking, no GNP probe aggregates could be noted on the surface of the NC membrane. After drying at 37°C, the antibody modified NC membrane was immersed into the liquid sample, as well as the addition of GNP probes. In another possible procedure, GNP probes could be added after the capture process. As shown in Figure S2, the results from these two procedures were compared. It can be seen that although the absorbance from 100 CFU/ml samples are similar, but in the procedure where GNP probes were added after the capture process, the absorbance from blank is stronger than the result from 100 CFU/ml samples and the result from blank in the procedure where GNP probes were presented in the capture process, which could be attributed to the increased nonspecific binding of GNP probes on the nitrocellulose membrane. Compared to the addition of GNP probes after capture, the detection results were better when the GNP probes were present in the capture process. For a better capture of target bacteria, the sample with the modified NC membrane was continuously mixed for a complete interaction between antibodies on NC membrane and target. Since the porous structure with micrometer scale pores provides a large surface and may influence the solution going through the membrane, mixing will play an important role in the capture of target bacteria. During mixing, the target bacteria

were captured on the membrane due to the specific interaction with antibodies, while the target bacteria will also be labelled with GNP probes. Due to the direct attachment of GNP probes on the NC membrane, there is an optimized amount of GNP probes resulting in better sensitivity. To verify the capture of target bacteria on NC membrane, membranes with antibody modified in a circular area were prepared. After reacting in TMB solution, as shown in Figure S3A correspondingly a circular area with green color was obtained on the NC membrane, indicating the capture of target bacteria. Similarly, if the whole NC membrane was modified with antibody, a green color was noted all over the membrane as shown in Figure S3B. After multiple washes to remove as much of the nonspecifically bound materials as possible, HRP was labelled onto GNP probes according to biotin-streptavidin interaction. Additive wash was performed to remove the nonspecifically bound HRP. Then NC membrane pad with the captured target bacteria labelled with GNP probes conjugated with HRP was immersed in TMB solution. Due to the TMB reaction catalyzed by the HRP the colored product can be recognized by naked eyes while the UV-vis spectra of the solution can be used for quantitative detection of target bacteria. Benefiting from the HRP catalysis, small increase in the GNP probe amount due to the captured target bacteria would be amplified in the final results. Although more HRP used in detection could increase the signal, the nonspecific interaction between HRP and NC membrane requires an optimization of the amount of HRP for best sensitivity.

According to the proposed detection method, typical results obtained are shown in Figure 1. It can be seen that the TMB solution changed into green color (photo as

Figure 1 inset). Due to the nonspecific attachment of GNP probes on antibody modified NC membrane, slight green color can be seen in the blank samples. For samples with 10 ml of 100 CFU/ml *E. coli* O157:H7, there is an obvious increase in color intensity as observed. It can be easily recognized based on the color difference by naked eyes between blank and samples with as low as 100 CFU/ml of target bacteria. To obtain a quantitative determination, UV-vis spectra of the reacted TMB solution were recorded. The intensity of the peak located at 661 nm, which was the maximum absorption of the colored product, was chosen to present the amount of target bacteria in samples, while to reduce the influence from the variation of spectral baseline, the absorption at 661 nm was normalized by subtracting the absorption at 535 nm. Our results show (Figure 1) that the proposed net fishing enrichment with HRP amplification of the presence of target bacteria enables detection by naked eyes while the UV-vis spectra will provide a quantitative assessment.

With NC membrane as the substrate for bacteria detection, one of the concern is the nonspecific binding. In our detection procedure, GNP probes and HRP would possibly nonspecifically attach to the NC membrane, therefore blocking is necessary for better detection. Serial concentrations of casein were used to block the NC membranes along with the modification of antibody. Since the detection results show that the concentration of blocking casein not only influenced the difference between blank samples and samples with target bacteria but also decreased the UV-vis spectral intensity, we presented the UV-vis spectra of detection results in Figure 2. Figure 2A shows the UV-vis spectra from the blank samples detected with NC membrane

blocked with no casein, 50, 500, and 5000 ppm casein. It can be seen that the concentration increase of casein used for blocking reduced the intensity of UV-vis spectra. The decrease in intensity because of more casein used for blocking suggested nonspecific interactions between NC membrane and HRP or GNP probes, since HRP and GNP probes later labelled with HRP during detection will catalyze the TMB reaction. The decrease in UV-vis spectra intensity according to casein concentration indicated that the nonspecific binding between NC membrane and HRP and GNP probes can be improved by simply increasing the casein concentration. However, comparison of detection results between blank samples and samples with 100 CFU/ml target bacteria (shown in Figure 2B, C, D and E) demonstrated a decrease in the difference between blank and 100 CFU/ml samples. To better illustrate the difference between the results from NC membrane blocked with various concentration of casein, the UV-vis spectra shown in Figure 2B, C, D and E was normalized based on the absorption of 535 nm in the spectrum of blank obtained from NC membrane without blocking (Figure 2Ba). Two possible reasons were proposed for the decrease in difference due to the increased amount of casein used for blocking. First, since the casein was applied on NC membrane as well as antibody, casein in high concentration may influence the conjugation of antibody on the NC membrane. Secondly, increased amount of casein on the NC membrane may encapsulate antibodies conjugated on NC membrane thus influencing the capture of target bacteria. Both of these are able to limit the capture of target bacteria, resulting in poor detection sensitivity. If the intensity of UV-vis spectra is too weak, as shown in Figure 2D and E, it will be hard

to see the color of the final solution by naked eyes. Therefore, considering the nonspecific binding, sensitivity and recognition, the presence of target bacteria by naked eyes, 50 ppm casein blocking was chosen for the following detection.

To achieve the best sensitivity, serial detection conditions were optimized. As described above, capture time, the amount of GNP probes and HRP would play an important role in increasing the difference in detection results between blank samples and samples with target bacteria. Figure 3 illustrated the optimization of these detection conditions with blank and samples with 100 CFU/ml. Since the increase in peak intensity due to the capture of target bacteria on the NC membrane compared with blank, the capture time determining the capture rate of target bacteria in samples is important for sensitivity. It can be expected that longer capture time will result in a higher capture rate. However, on the other hand, because of the nonspecific attachment of GNP probes on the NC membrane, longer interaction time will cause more GNP probes to be directly anchored to the NC membrane, resulting in a stronger intensity of the blank sample and thus a decrease in the difference between blank and samples with target bacteria. Detection with capture time from 1 hour to 4 hours were performed and the obtained differences are shown in Figure 3A, where $\Delta\text{Abs.}$ is defined as: $\Delta\text{Abs.} = \text{Abs.} - \text{Abs.}_0$, where Abs. is the normalized absorption from the samples with target bacteria and Abs._0 is the normalized absorption from blank samples. It can be seen that when the capture time increased from 1 hour to 2 hours, an increase of $\Delta\text{Abs.}$ was observed, which could be attributed to the increased capture rate of target bacteria. When the capture time further increased to 3 or 4 hours, the

Δ Abs. decreased, possibly assigned to an increase in nonspecific attachment of GNP probes and thereby stronger Abs. from blank samples. The optimization of capture times indicated that capture for 2 hours would provide the best sensitivity. The influence due to nonspecific attachment of GNP probes is also shown in the optimization of GNP probe amount. As shown in Figure 3B, serial concentration of GNP probes were used to detect blank and 100 CFU/ml of samples. The increase in the used GNP probes from 1 μ l to 2.5 μ l slightly improves the Δ Abs., suggesting that 2.5 μ l should be enough for detection of 100 CFU/ml samples. Further increase in the amount of used GNP probes could decrease the Δ Abs. even to be negative, which could be attributed to the nonspecific attachment of GNP probes on NC membrane. Meanwhile, the negative Δ Abs. obtained with 10 μ l GNP probes suggested that the captured target bacteria covered part of the NC membrane surface thus limiting the direct attachment of GNP probes on the NC membrane. Per the optimization, 2.5 μ l of GNP probes was used for detection. Because the final signal was generated based on the HRP catalyzed TMB reaction, an increase in the amount of HRP linked to GNP probes labelled on captured target bacteria will increase the difference between UV-vis spectra from blank and samples with target bacteria. However, an increase in HRP could induce some problems: i) too many HRP could increase nonspecific binding of HRP on NC membrane; ii) because the HRP catalyzed TMB reaction rate is high, too many HRP molecules could induce a larger deviation in detection results since it is not practical to keep the reaction time precisely the same. The results from detection with different amount of HRP is shown in Figure 3C. It can be seen that an

increase of HRP results in an improved $\Delta\text{Abs.}$ from 1 to 10 μl . However, as illustrated, 10 μl HRP resulted in a large deviation of detection signal. Considering the sensitivity and reproducibility, 5 μl HRP was used for detection based on the results of optimization.

Based on the optimized detection conditions, to investigate the quantitative response of the proposed method integration of net fishing enrichment and HRP amplification, serial concentration of target bacteria, *E. coli* O157:H7, from 100 CFU/ml to 100000 CFU/ml in PBS buffer were tested. The obtained results were shown in Figure 4. It can be seen that the detected signal increase in proportion of *E. coli* O157:H7 concentration. As low as 100 CFU/ml *E. coli* O157:H7 can be detected and a linear response was established ($R^2=0.981$). It is noted that the signal increase from 100 CFU/ml to higher is not as much as that between blank to 100 CFU/ml, which could be assigned to the limited capture of target bacteria at high concentration. As illustrated in Scheme 1, most of the pores on NC membrane are less than 10 μm , therefore the capture of target bacteria should occur on the surface of the NC membrane and may influence the introduction of bacteria into the NC membrane. Therefore an increase in signal due to high concentration of target bacteria is not as much as that with low concentration. To test the feasibility of the proposed method for monitoring food samples, juice was chosen as real samples where the contaminations of *E. coli* O157:H7 could potentially occur. *E. coli* O157:H7 was purposefully inoculated in pineapple juice purchased from the local supermarket and used as a substrate to validate the proposed method. To reduce the influence from the

thickening agents and other materials in the juice for target detection, the juice was diluted with 10 mM PBS in 1:1 ratio before detection. Figure 5 shows the detection results from the diluted pineapple juice and inoculated with *E. coli* O157:H7. 400 CFU/ml of *E. coli* O157:H7 is the concentration before dilution, thus the detected concentration of *E. coli* O157:H7 is estimated to be 200 CFU/ml in diluted pineapple juice. Though an increase in signal is not as much as that in PBS, as low as 200 CFU/ml of *E. coli* O157:H7 in the diluted juice can be recognized with the proposed method by integrating net fishing and HRP amplification, highlighting the feasibility of the proposed method for monitoring pathogens in liquid samples.

Conclusion

Herein we proposed a detection method based on the integration of net fishing enrichment of target bacteria from liquid samples and HRP amplification of signal. Net fishing is a unique enrichment method different from the previous culture-based, filtration or magnetic nanoparticle-based enrichment strategies. NC membrane with a porous structure was chosen as a substrate, and antibodies were conjugated to the membrane to capture the target bacteria in samples in 2 hours. The proposed enrichment strategy is faster than culture-based enrichment while the specific recognition due to antibody reduced the nonselective enrichment during filtration. GNP probes labelled with HRP were used as probes to generate signal based on catalyzed TMB reaction. With the amplification of HRP, improved detection sensitivity could be achieved. After optimization of capture time, the amount of GNP probes and HRP, we show that as low as 100 CFU/ml of *E. coli* O157:H7 can be

detected and a linear response from 0 to 10^5 CFU/ml could be achieved. Finally, we demonstrated detection of pathogens in pineapple juice to show the feasibility of our method in food samples. The developed target enrichment strategy with signal recognizable by naked eyes would be a viable option for routine monitoring of pathogens in large volume samples requiring enrichment before detection.

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Figure caption

Scheme 1. Illustration of preparation of NC membrane and detection of target bacteria in liquid samples.

Figure 1. UV-vis spectra of detection results from blank (a) and 100 CFU/ml *E. coli* O157:H7 in 10 mM PBS (b), the inset (photo) shows the color of the detection results.

According to the color analysis of the photo, the mean value of the grayscale of blank and 100 CFU/ml sample in the photo was 218 and 193, respectively.

Figure 2. UV-vis spectra of the detection results with NC membrane blocked with serial concentration of casein (A) and the results from blank (a) and 100 CFU/ml *E. coli* O157:H7 in 10 mM PBS (b) with NC membrane blocked with no casein (B), 50 ppm (C), 500 ppm (D) and 5000 ppm (E) casein.

Figure 3. Optimization results of capture time (A), GNP probe amount (B) and HRP amount (C).

Figure 4. Detection results from samples with serial concentrations of *E. coli* O157:H7 in 10 mM PBS.

Figure 5. Detection of *E. coli* O157:H7 in pineapple juice diluted with PBS in 1:1 ratio.

Scheme 1.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

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