Development and Evaluation of Colloidal Gold Lateral Flow Immunoassays for Detection of \textit{Escherichia Coli O157} and \textit{Salmonella Typhi}

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Abstract. Lateral flow immunoassays (LFIs) are rapid and simple detection methods for detecting bacterial contamination in food and drinking water such as \textit{Escherichia coli O157} and \textit{Salmonella Typhi}. \textit{Escherichia coli O157} can cause haemorrhagic colitis, diarrhoea and haemolytic uraemic syndrome, whereas \textit{Salmonella Typhi} is responsible for typhoid fever in human. Colloidal gold nanoparticles (AuNPs) synthesized using citrate reduction method is commonly used as labels for LFIs. However, in this study, AuNPs synthesized using the seeding-growth method was evaluated as label in LFIs. The 15 nm AuNPs seed was first synthesized using the citrate reduction method followed by growth to 40 nm AuNPs using mild reduction agent, hydroxylamine. This monodispersional AuNPs was then conjugated with anti-gram-negative endotoxin antibody at 3 μL/mL. The conjugate then immersed to conjugate pad of glass fiber at OD 10. Two sets of nitrocellulose membrane (NC) were used to immobilize anti-\textit{E. coli O157} antibody as the test dot at 1.7 mg/mL and anti-mouse IgG antibody as the control dot at 1.0 mg/mL. Another set of NC immobilized with 1.0 mg/mL anti-\textit{Salmonella sp.} antibody as the test dot and the same control dot as first NC set. Spiked water and milk sample was prepared by adding 10-fold dilution ATCC stains of \textit{E. coli O157} and \textit{Salmonella Typhi} to water and milk samples. LFIA to \textit{E. coli O157} in water and milk sample was able to detect as low as $7.8 \times 10^5$ and $3 \times 10^6$ CFU/mL, respectively, whereas LFIA to \textit{Salmonella Typhi} was able to detect as low as $3 \times 10^8$ and $3 \times 10^7$ CFU/mL in water and milk sample, respectively. Specificity for both strips sets was 100%. This method is very useful for monitoring bacterial contamination in food and drinking water towards ensuring food and water safety.

1. Introduction

Current trends in nutrition and food technology have attracted attention of food microbiologist to ensure food supply is safe before entering the market. Bacterial pathogens encountered to human illness in the last
decades through consumption of undercooked or minimally processed ready-to-eat meats, dairy products or fruits and vegetables [1-3]. Two of most commonly food borne pathogens are *Escherichia coli* O157 and *Salmonella* Typhi.

*Escherichia coli* O157:H7 is one of an *Enterohemorrhagic E. coli* (EHEC) that causes diarrhea associated with abdominal cramps, hemolytic uremic syndrome, hemorrhagic colitis, thrombotic thrombocytopenic purpura and presentation of mild fever [4, 5]. *E. coli* O157:H7 infection is hardly found in Malaysia but it is required for precaution by screening all imported foods and beverages. *Salmonella* Typhi on the other hand is responsible for enteric fever causes by typhoid fever [6]. The above bacterial infection if not treated rapidly can increase rate of morbidity and mortality [7]. The conventional and traditional method for detection of these bacterial are culture method, Polymerase Chain Reaction (PCR), enzyme-linked immunosorbent assay (ELISA) and immunological techniques [8]. Unfortunately, these methods require technical expertise, expensive equipment, laborious protocol and impractical for on field detection. Thus, it is a must to develop a rapid, simple and sensitive method for detection of these food borne pathogens.

The lateral flow immunoassay strips (LFIA) is a paper-based biosensor device applying a modern version of immunoassay with advantages of rapid, low limit of detection, high sensitivity, good specificity, low manufacturing cost, less sample operation volume, robustness, user-friendly format and no complicated equipment needed [9]. The LFIA strips used nanomaterials as label which improve their performance for various applications [9, 10]. Colloidal gold nanoparticles (AuNPs) is the most widely used label owing to easy synthesis and modification, stable with time, size-tunable, biocompatible and have an intense red colour that easy to be detected even by naked eye or usually using a colour reader to achieve better detection limit [9].

Citrate reduction method is the most reported method for the synthesis of AuNPs with different size by using trisodium citrate as the reducing agent [5, 10-12]. However, this method hardly produces monodisperse spherical AuNP when the size exceeds 30 nm [13]. Nearly all AuNPs based LFIA use 40 nm in size, thus this method seems to be unsuitable. Moreover, AuNPs synthesized by this method requires more antibody to be stable due to unspecific interaction of the AuNPs surfaces with the sites of the antibody and therefore increasing the production cost of LFIA [14].

To overcome these problems, the seeding-growth method was used to synthesis AuNPs. In this method, smaller particles known as seeds can be grown into larger particles in controlled manner by using hydroxylamine (NH$_2$OH) as a mild reducing agent that reduces and prevents the formation of new nuclei to allow the growth process [15, 16]. Thus, in this work, 40 nm AuNP was synthesized by seeding-growth method to produce monodisperse spherical AuNP as a label in LFIA. Then, the performance of this LFIA for detection of *Escherichia coli* O157 and *Salmonella* Typhi in spiked water and milk samples was evaluated accordingly.

2. Experimental

### 2.1 Synthesis and Characterization of Colloidal Gold Nanoparticle (AuNP)

40 nm colloidal AuNPs was prepared by using the seeding-growth method as reported previously [14]. The prepared seeds then were grown to 40 nm AuNPs. About 4 mL seed was added to 50 mL deionized water while stirring followed by the addition of 1 mL hydroxylamine as a mild reducing agent to the solution. Then, 1% (w/v) of chlorouaaic acid was added dropwise. The synthesized AuNPs was characterized using UV-Vis-NIR spectrophotometer (Model UV-3600, Shimadzu), particle size analyzer (Model ZEN 3600, Nanoseries, Malvern Instruments) and transmission electron microscopy (TEM) (Model FEI CM12, Version 3.2 image analysis system, 120 kV).
2.2 Conjugation of 40 nm AuNPs with Antibody

The AuNPs was first adjusted to pH 7 with 0.2 M K₂CO₃. Volume of anti-gram-negative endotoxin antibody (GNE) was optimized to stabilize AuNPs. The volume of GNE that retained the original red wine colour of AuNPs after addition of 10% sodium chloride (NaCl) was chosen. The optimized volume was added into AuNPs. After incubated at room temperature for 30 min, 1% (w/v) of bovine serum albumin (BSA) was added to the conjugate and centrifuged at 10000 rpm for 10 min. Supernatant was then removed and this step was repeated twice. In the last washing, pellet was re-suspended in 1% BSA. This conjugate then was characterized by using UV-Vis-NIR spectrophotometer and optical density (OD) was measured.

2.3 Preparation of LFIA Strips

Two sets of LFIA strips were prepared, one set for detection of *E. coli* O157 and another one for detecting *Salmonella* Typhi. An HF90 nitrocellulose membrane was used to immobilize capture antibodies. For *E. coli* O157 LFIA strips, 0.5 μL at 1.7 mg/mL of anti-*E. coli* O157 antibody was pipetted at the lower part of NC membrane as the test dot. Anti-*Salmonella* sp. antibody was pipetted at 1.0 μL of 1.0 mg/mL as the test dot for *Salmonella* Typhi LFIA strips. Both sets were pipetted with 1.0 μL at 1.0 mg/mL of anti-mouse IgG antibody at upper part on the NC membrane as the control dot. The NC membrane was then dried for 2 h at 37 ºC. Blocking solution (50% v/v) of western blot solution in 0.01 M TBS pH 7.5 was flowed to NC membrane. This blocking solution was used to block the free site of the membrane. Blocked NC membrane then was re-dried overnight at 37 ºC.

The conjugate pad was prepared to hold AuNPs-GNE conjugate. The AuNPs-GNE conjugate was diluted in solution containing 0.2 mM Boric buffer, 1% BSA and 5% trehalose to be at OD 10. Glass fiber as conjugate pad was pipetted with 30 μL of the prepared OD 10 AuNP-GNE conjugate. This conjugate pad then dried overnight at 37 ºC. Sample pad from cellulose material was left untreated. Complete LFIA strips were then formed by assembled conjugate pad, NC membrane, sample pad and absorbance pad together.

2.4 Samples Preparation, Visual Limit of Detection (LOD) and Cross-Reactivity Testing of LFIA

American type culture collection (ATCC), ATCC® 43888™ of *E. coli* O157:H7, ATCC® 10536™ of *E. coli*, ATCC® 12027™ of *Shigella flexneri* and ATCC® 14028™ *Salmonella* Typhi were grown in trypticase soy broth and adjusted to McFarland standard 1.0 (approx. 3 x 10⁸ CFU/mL). The ATCC of *E. coli* O157 and *Salmonella* Typhi were diluted in 10-fold from 3 x 10⁸ to 3 x 10¹ CFU/mL. The dilution then was spiked to sterile water and milk. ATCC of *E. coli* and *Shigella flexneri* at 3 x 10⁸ were also spiked to sterile water and milk for specificity testing.

The LFIA strips were pipetted with the spiked sample on the sample pad. The sample was then flowed to the conjugate pad, NC membrane and finally to the absorbent pad by capillary action. *E. coli* O157 and *Salmonella* Typhi were pipetted to LFIA containing anti-*E. coli* O157 and anti-*Salmonella* sp. antibody respectively. Uninoculated sterile water and milk were used as negative control and flowed to both LFIA sets. After that, a drop of Chase buffer containing 0.035% Tween-20 was used to wash LFIA for removing background stain. Intensity, present or absent of control and test dots were observed for each set.

3. Results and Discussion

3.1 Characterization of Colloidal AuNPs

The colloidal AuNPs was successfully synthesized using the seeding-growth method. The size of 15 nm AuNPs seed and 40 nm AuNPs were confirmed from TEM images and particle size analyzer (Figures 1 and
This result was comparable to previous study where AuNPs synthesized by the seeding-growth method was highly monodispersed with uniform size [14]. Normal distribution histogram with average 17 nm for AuNP seed and 42 nm for 40 nm AuNPs (Figure 2a – b) was obtained from particle size analyzer. The size obtained by particle size analyzer is slightly bigger than TEM because particle size analyzer measures hydrodynamic size of the particle which include thickness of the electrical double layer (solvation shell) as well as the presence of adsorbed substance such as stabilizer [17]. The UV-Vis-NIR spectroscopy analysis showed the maximum peak at absorbance (OD) around 1 for both seed and 40 nm AuNPs (Figure 2c). The maximum peak for AuNPs seed was at wavelength ($\lambda_{max}$) 519 nm whereas for 40 nm AuNPs at 527 nm. This also confirmed that AuNPs seed is smaller than 40 nm AuNPs because the maximum peak was red shifted form AuNPs seed to 40 nm AuNPs [14, 17].

![Figure 1. TEM images of (a) 15 nm AuNPs seed, and (b) 40 nm AuNPs](image1.png)

![Figure 2. Histograms of size distribution by intensity of particle size analyzer for (a) 15 nm AuNP seed, (b) 40 nm AuNP, and (c) UV-Vis-NIR spectra for 15 nm AuNPs seed and 40 nm AuNPs](image2.png)

### 3.2 Characterization of Antibody-AuNP Conjugates

Stable conjugate was achieved when the entire surface of AuNPs was covered by sufficient amount of antibody. Unstable conjugate agglomerated when 10% NaCl was added thus change the color of the solution. This happened when negative ionic double layers around AuNPs was broken and aggregated [14, 18, 19]. The presence of enough antibody around AuNPs prevented flocculation. In this work, 1 mL of 40 nm AuNPs at pH 7 needed 3 μL anti-GNE antibody to stabilize.

Successfulness of conjugation was confirmed by comparing the UV-Vis-NIR spectra before and after conjugation. The peak of UV-Vis-NIR spectra after conjugation was red shifted from 527 nm to 532 nm which confirmed the attachment of antibody to the surface of AuNP. The concentration of the conjugates was taken by determine the absorption value as optical density (OD). In this work, the OD of 40 nm AuNP after conjugated was at OD 24.

### 3.3 LFIA Test

The LFIA test was interpreted by observing the formation of red color dot/s on the NC membrane. If the sample contains *E. coli* O157 and/or *Salmonella* Typhi, the bacteria binds to the gold conjugated anti-GNE antibody in the conjugate pad and formed antibody-antigen complex. The antibody-antigen complex then moved by capillary action to the test dot. The reaction then occurred where the complex was captured by
their respective specific anti-*E. coli* O157 or anti-*Salmonella* sp. antibody that was immobilized as the test dot on the NC membrane. Accumulation of many complex at the test dot formed a visible red dot. The visibility and intensity of red dot were proportional to the amount of bacterial in the sample [10, 20]. The excess AuNPs conjugated anti-GNE antibody then kept flowing and reached the control dot. In here, AuNPs conjugated anti-GNE antibody which is a mouse IgG was captured by anti-mouse IgG antibody that was immobilized as the control dot on the NC membrane. The control dot then appeared as the second dot on the upper part of NC membrane which then validated the test. Negative result was interpreted when only the control dot appeared, whereas if no control dot appeared either present or absent of the test dot, the test is considered as invalid. The reaction occurred within 15 min. Chase buffer as wash solution was applied after the reaction to wash away the red background on NC membrane. Tween-20 in the Chase buffer functions as detergent because the background residual contained both soluble and insoluble matrix.

### 3.4 Visual LOD and Cross-Reactivity Testing of Spiked Samples

In this study, visual LOD and cross-reactivity of spiked samples of two commonly food borne pathogens, *Escherichia coli* O157 and *Salmonella* Typhi in sterile deionized water and packed full cream milk samples (purchased from local mart) was determined. Four sets of LFIA strips each to detect *E. coli* O157 (Figure 3a – b) and *Salmonella* Typhi (Figure 3c – d) in water and milk was used. For detection of *E. coli* O157 spiked in water and milk samples, the lowest detectable of LFIA strip was $7.8 \times 10^5$ CFU/mL (Figure 3a-8) and $3.0 \times 10^6$ CFU/mL (Figure 3b-2), respectively. The LFIA strip also was able to detect as low as $3.0 \times 10^8$ CFU/mL (Figure 3c-1) and $3.0 \times 10^7$ CFU/mL (Figure 3b-1) of *Salmonella* Typhi spiked in water and milk sample, respectively.

![Figure 3. LFIA strips for detection of: (a) *E. coli* O157 in water sample, (b) *E. coli* O157 in milk sample, (c) *Salmonella* Typhi in water sample and (d) *Salmonella* Typhi in milk sample](image)

The visual LOD or sensitivity of LFIA strip to *E. coli* O157 both in spiked water and milk samples was comparable with the previous studies [21-23]. The result also showed that the LOD for spiked milk sample is lower than water sample. Milk sample used in this work was full cream milk which contained high amount of lipid that may interfere the reaction of this test [24, 25]. The milk sample also needed to be diluted with water before spiked with bacterial and pipetted to LFIA strips. The undiluted milk sample was unable to flow in LFIA strip. However, visual LOD of LFIA strip to *Salmonella* Typhi was low. This could be due to low concentration of anti-*Salmonella* sp. antibody used as the test dot compared to the concentration used for anti-*E. coli* O157.
Overall, the visual LOD for both bacterial can be improved by implementing the enrichment method as previous studies [5, 21, 22]. However, adding this method will prolong the turnaround time for the test. This is because enrichment method needs 18 – 24 hour of sample incubation. Furthermore, additional reagents are also needed as enrichment medium. Thus, this will increase cost as well as labor cost and impractical for on field testing application.

In term of specificity, no cross-reactivity was detected for both strips when other bacterial were used as samples (Figure 3a-10,11,12; 3b-8,10,11; 3c-7,8 and 3d-8,9,10). Unfortunately, for anti-\(E.\ coli\) O157 strip, very pale red color was detected when \(E.\ coli\) non O157 was used in the water sample (Figure 3a-11). This is due to very close relation of genetic material between \(E.\ coli\) O157 and \(E.\ coli\) non O157 strains [26]. However, this false positive can be reduced by washing step using Chase buffer. Besides, all test strips could produce result within 10 – 15 min which can be considered as rapid test and comparable to the previous studies [5, 12, 21, 23].

4. Conclusions

Monodisperse 40 nm AuNPs was successfully synthesized using the seeding-growth method. GNE antibody was also successfully conjugated with 40 nm AuNPs at pH 7. The LFIA strip was successfully optimized and used for detection of food-borne pathogen (\(E.\ coli\) O157 and \(Salmonella\) Typhi). This LFIA could detect bacterial in water and milk spiked samples as low as \(7.8 \times 10^5\) CFU/mL. Both LFIA strips have higher specificity which only detect \(E.\ coli\) O157 and \(Salmonella\) Typhi but not to other cross related Enterobacteriaceae. The assay time was 10 – 15 minutes and could be considered as rapid test.

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