Development of Starch-Polyvinyl Alcohol Films-based pH indicator for Detection of Penicillin G Residue in Raw Milk

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Abstract. The detection of residual penicillin G (Pen G) in raw milk is very crucial to prevent the adverse effect from the allergy of Pen G to dairy consumer. However, the conventional detection methods require highly expensive instruments, high-skill technicians and complicated sample preparation process. In this research, a Lab-on-a-Disc (LOAD) to detect the residual Pen G in raw milk was introduced. The LOAD (φ: 9.0 cm) consisted of 10 micro-channels with the maximum volume of 200 µL was fabricated by laser machining of PMMA sheets. The 8.0 µm-thick polyvinyl alcohol, PVA (0.03125 % w/v) mixed with corn starch at a ratio of 3:7 (w/v) and bromocresol purple (BCP) pH indicator (0.025 % w/v) film that used as a Pen G indicator was prepared by hydrogel method and coated on the micro-channels by drop casting method. After the LOAD was rotated at 5000 rpm for 5 min at 25 °C, the BCP-(S-PVA) indicator film with the solubility of 93.56 % was dissolved in a raw milk mixed with Pen G (4.0 ng/mL) and the color of BCP-(S-PVA) film has changed from dark violet to light violet. The detection limit of BCP-(S-PVA) film to detect the Pen G in raw milk that obtained from the linear dose response curve (R²: 0.9630) is 0.17 ng/mL. Therefore, the LOAD with the BCP-(S-PVA) pH indicator is the fast and accurate promising method to determine the residual Pen G in raw milk at room temperature.

1. Introduction

Raw milk and dairy products are known as rich sources of essential nutrients in human diet such as high-quality proteins, vitamins, minerals, and essential micro-nutrients including calcium, magnesium, potassium, zinc, and phosphorus [1, 2]. In addition, raw milk can also provide nutrient-rich mediums which is a suitable environment for micro-organism growth [3, 4]. Thus, several antibiotic agents were introduced to treat against rapid growth of micro-organism [5, 6]. Among the antibiotic, Penicillin is one of the most popular β-lactam antibiotic due to the presence of a β-lactam ring in the...
molecular structure [7]. It is widely used for treatment of bovine mastitis, lung infections, and bacterial arthritis in veterinary medicine [8]. However, it was reported that approximately 10% of the patients around the world are allergy to penicillin. This is due to an abnormal reaction of their immune system to the penicillin in an antibiotic drug [9, 10]. Therefore, the Food and Drug Administration (FDA) announced the regulation of penicillin for human use to prevent an adverse effect [11]. In parallel, several methods have been developed to determine the amount of Pen G in raw milk such as chromatographic methods [12, 13], immunoassays [14] and rapid test kits [15]. These methods require highly expensive instruments, high-skilled technicians and complicated sample preparation process.

Therefore, a LOAD which is a unique microfluidic platform that utilizes centrifugal force to pump and drive liquids. This offers many benefits for on-site testing devices because it eliminates the need for connections to multiple pumps and complex tubing connections. Hence, the LOAD is one of a promising candidate method to detect a residue Pen G in raw milk due to its rapid reaction time, small volumes of sample and reagents, user-friendly, and low-cost [16]. However, the LOAD is required a chemical or film indicator that insert into the micro-channel to react with the residual Pen G in raw milk. The color change of the indicator by the chemical reaction is one of the popular method to detect the concentration of the target substance.

In this research, poly (vinyl alcohol) or PVA, which is a synthetic non-toxic biodegradable polymer with good solubility in water, was chosen as a polymeric film that coated onto the microfluidic channel to entrap the reagents on the LOAD. This PVA film can prevent the losing and spilling of solution during transportation and characterization in the LOAD. However, the lack of long-term stability and low mechanical strength are limited utilizing of a PVA film. It has been reported that blending the PVA with starch (S-PVA) can improve the stability, mechanical properties and biocompatibility of the PVA-based films [17]. This is due to the starch that highly contains hydroxyl (-OH) groups, which tend to form inter-molecular and intra-molecular hydrogen bonds that significantly improve the stability and integrity of S-PVA film [18, 19]. Then the suitable pH indicator such as bromocresol purple (BCP) with the pH range of 5.4 to 6.8 is mixed with PVA and S-PVA films. The BCP-(PVA) and BCP-(S-PVA) films can be used to detect the residue Pen G antibiotic in raw milk. When the BCP-(PVA) and BCP-(S-PVA) films are dissolving in a raw milk, the pH indicator is releasing into the solution. Then the color change of the solution, which is due to the pH change from the enzymatic hydrolysis of penicillin to penicilloic acid will be monitored. The color of the solution can be used to indicate the amount of the residue Pen G in raw milk.

The aim of this research is to study the effects of the concentration of PVA and S-PVA to the degree of solubility of those films. The optimized concentration of the BCP pH indicator to the dose response curve of BCP-(PVA) and BCP-(S-PVA) films to detect the amount of Pen G antibiotic in raw milk were evaluated. Moreover, the performance of the BCP-(PVA) and BCP-(S-PVA) films integrated with the LOAD to detect the residue Pen G antibiotic in raw milk were investigated.

2. Experimental Procedure

2.1. Preparation of PVA and S-PVA films

In this research, the PVA solution (Sigma-Aldrich, Missouri, USA) was used as an initial substance to form a PVA film by dissolving it in the distilled water and stirred at 95 °C for 30 min [20]. The concentration of PVA solution was varied from 0 to 10 % w/v. The S-PVA film was prepared by mixing the corn starch with the PVA solution at a ratio of 3:7 (v/v) and dissolved in distilled water and stirred at 95 °C for 30 min. The hydrogel method was used to prepare PVA and S-PVA films and incubated at 70 °C for 3 h. The degree of solubility of PVA and S-PVA films were carried out by mixing food coloring dye (Red color) during the film copolymerization. After that PVA and S-PVA films with different concentration of 0 to 10 % w/v were immersed in deionized (DI) water at room temperature (25 °C) for 0, 5, 10 and 30 min. The food coloring that released after PVA and S-PVA films dissolved in DI water were measured at the wavelength (λ) of 532 nm and the percentage of film solubility was determined according to the equation (1), which was adapted from [20].
Percent Solubility = \frac{OD_{solution} - (OD_{solution} - OD_{film dissolved})}{OD_{solution}} \times 100 \quad (1)

where the optical density (OD) solution represents the absorbance of food coloring dye (Red color) in solution and OD film dissolved represents the absorbance of the food coloring dye releasing after PVA and S-PVA dissolved in DI water.

The field-emission scanning electron microscope (FE-SEM, Hitachi S-4700) was used to determine the thickness and the surface topology of PVA and S-PVA films. Note that 2.0 nm-thick gold and palladium (Au/Pd) film was deposited onto the PVA and S-PVA films to prevent a charging effect.

2.2. LOAD components and fabrication

In this study, the LOAD with a diameter (φ) of 9.0 cm and the length between the center of the disc to the end of the channel that located at the edge of the disc was 42.0 mm. The LOAD consists of ten sets of channels, chambers, inlet holes, and outlet air vents. Each channel has a maximum volume of 200 µL (Fig. 1(A)). The LOAD comprises of five different layers which are inlet hole (layer-1), fluid chamber (layer-3), base layer (layer-5) and two pressure sensitive adhesive (PSA) layers (layer-2 and layer-4). All the micro-patterns of the LOAD including micro-channels, micro-chambers, loading holes and air vents, were fabricated on Polymethyl Methacrylate (PMMA, acrylic) sheet using laser machining. Then, the PMMA layers were cleaned by 2-propanol and blown dried with pure N₂ gas. Later that the PMMA, PE and PSA layers were manually attached together to assembled the LOAD (Fig. 1(B)). Finally, the BCP-PVA and BCP-(S-PVA) films were coated into the micro-channels of the LOAD by using a drop casting method (Fig. 1C).

![LOAD Design](image)

(A.) LOAD design with 10 sets of micro-channels, (B.) LOAD components that comprises of five different layers, and (C.) Cross-sectional view of BCP-PVA and BCP-(S-PVA) films coated on the micro-channel of LOAD.

2.3. Optimization of BCP concentration and dose response curve

To optimize the BCP concentration, the BCP pH indicator (Merck, Massachusetts, United States) was added into PVA and S-PVA solution by varied the concentration of 0.03125 to 0.1 % w/v. The hydrogel method was used to prepare the BCP-PVA and BCP-(S-PVA) films onto the micro-channels of the LOAD. The BCP-PVA and BCP-(S-PVA) films were also prepared in 96-wells plate. Then the raw milk (from PSU and KU, Thailand) mixed with 4.0 ng/mL of Pen G (Sigma-Aldrich, Missouri, USA) was introduced into micro-channels of LOAD and 96-wells plate and incubated at room temperature (25 °C) for 30 min. Note that the incubation of 30 min is excess the time that the BCP-PVA and BCP-(S-PVA) films requires to completely dissolved in DI water. Then the LOAD was rotated at 5000 rpm for 5 min (25 °C).

In case of the dose response curve, the Pen G with different concentration of 1 to 100 ng/mL was prepared and separate spiked into raw milk. Each Pen G concentrations were subsequently introduced into the BCP-(S-PVA) film on both LOAD and 96-wells plate. After that penicillinase was added into those samples, the LOAD was rotated at 5000 rpm for 5 min (25 °C). The color change of
BCP solution in the BCP-(S-PVA) film that correlated to the end-point reaction between Pen G and penicillinase in raw milk was monitored by using spectroscopy method (Azure AC3000 microplate reader, Dublin, United States) for the samples in the 96-wells plate and using the visual inspection for the samples in the LOAD.

3. Results and discussion

3.1. The degree of solubility of PVA and S-PVA films

The results in Table 1 and Table 2 showed the degree of solubility of PVA and S-PVA films with different PVA concentrations of 0.03125 to 10 % w/v when the solubility time was varied from 0 to 30 min. Note that the S-PVA film was prepared by mixing the corn starch with the PVA solution at a ratio of 3:7 (v/v). It was found that when the concentration of PVA has increased from 0.03125 to 10 % w/v, the solubility of both PVA and S-PVA have decreased as the results shown in Fig. 2(A) and Fig. 2(B), respectively. When the solubility time has increased, the degree of solubility of PVA and S-PVA films have increased. This is because those films have more soluble when the reaction time has increased. The solubility of S-PVA film is higher than PVA film. This is due to the starch properties in S-PVA film, which are high polar polymers rich with hydroxyl groups, tend to form inter-molecular and intra-molecular hydrogen bonds after copolymerization which can improve stability and integrity of S-PVA film [18, 19]. Moreover, longer solubility time of S-PVA film compared to PVA-film is due to the S-PVA film (8.0 ± 2.0 µm) is thicker than the PVA film (4.0 ± 2.0 µm) as shown in the insets SEM images of Fig. 2(A.) and Fig. 2(B.), respectively.

Based on the aforementioned results, the S-PVA film is suitable to be used as a polymeric film on a LOAD due to its much more stability and integrity than PVA film. The optimized concentration of PVA in the S-PVA film was 0.03125 % w/v because it has a highest degree of solubility of 93.56 % at the solubility time of 5 min. Whether the degree of solubility of S-PVA film has increased from 93.56 % to 100 % when the solubility time has increased from 5 to 30 min. However, the shorter solubility time is required for the rapid testing of LOAD. Therefore, the S-PVA film with the PVA concentration of 0.03125 % w/v with a solubility time of 5 min is the optimized condition for the polymeric film on a LOAD.

Table 1 The degree of solubility of PVA film with different PVA concentrations of 0.03125 to 10 % w/v when the solubility time was varied from 5 to 30 min. Note that the film thickness is 4.0 ± 2.0 µm.

| Solubility time (min) | 0.03125 % w/v | 0.0625 % w/v | 0.125 % w/v | 0.25 % w/v | 0.5 % w/v | 1 % w/v | 2 % w/v | 5 % w/v | 10 % w/v |
|---------------------|---------------|--------------|-------------|------------|----------|--------|--------|---------|---------|
| 5                   | 95.06 %       | 92.73 %      | 75.59 %     | 73.57 %    | 53.60 %  | 50.99 %| 35.70 %| 8.77 % | 8.93 %  |
| 10                  | 99.77 %       | 95.47 %      | 92.26 %     | 85.63 %    | 76.69 %  | 68.42 %| 48.35 %| 11.63 %| 13.05 % |
| 30                  | 100.00 %      | 99.96 %      | 99.90 %     | 99.52 %    | 82.38 %  | 75.34 %| 53.69 %| 23.64 %| 17.58 % |

Table 2 The degree of solubility of S-PVA film (corn starch: PVA = 3.7 (v/v)) with different PVA concentrations of 0.03125 to 10 % w/v when the solubility time was varied from 5 to 30 min. Note that the film thickness is 8.0 ± 2.0 µm.

| Solubility time (min) | 0.03125 % w/v | 0.0625 % w/v | 0.125 % w/v | 0.25 % w/v | 0.5 % w/v | 1 % w/v | 2 % w/v | 5 % w/v | 10 % w/v |
|---------------------|---------------|--------------|-------------|------------|----------|--------|--------|---------|---------|
| 5                   | 93.56 %       | 92.31 %      | 89.42 %     | 86.27 %    | 69.24 %  | 64.36 %| 53.89 %| 41.76 %| 14.08 % |
| 10                  | 95.72 %       | 96.86 %      | 92.31 %     | 89.12 %    | 84.50 %  | 67.12 %| 60.89 %| 53.17 %| 23.01 % |
| 30                  | 100.00 %      | 98.23 %      | 95.27 %     | 94.78 %    | 90.95 %  | 90.04 %| 80.85 %| 76.11 %| 40.53 % |
3.2. The Optimized concentration of BCP and dose response curve

By using the BCP-(S-PVA) film with the PVA concentration of 0.03125 % (w/v), the color of raw milk mixed with Pen G of 4.0 ng/mL (a spiked sample) has changed from white to dark purple when the BCP concentration has increased from 0 to 0.0625 % (w/v) after dissolved for 5 min as shown in the insets of Fig. 3(A.). The color change is correlated to the pH range of a spiked raw milk with Pen G of 4.0 ng/mL. Note that the pH range of BCP is 5.4 to 6.8, which facilitates the color change from yellow to purple. Figure 3(A.) showed that when the concentrations of BCP in the BCP-(S-PVA) film were 0.00625 %, 0.0125 %, 0.025 % and 0.05 % w/v, the absorbance of BCP-(S-PVA) film at λ of 595 nm were 0.09 ± 0.01, 0.38 ± 0.01, 0.76 ± 0.01 and 0.81 ± 0.01, respectively. This means, the absorbance of BCP-(S-PVA) film was directly proportional to the concentration of BCP. Whether the concentration of BCP-(S-PVA) film which higher than 0.025 % (w/v) can obtain a high absorbance value, those BCP concentrations were not practical for the LOAD due to too dark color was difficult to detect the change of the concentration of Pen G in raw milk. Too low concentration of BCP was also difficult to detect the change of the concentration of Pen G in raw milk due to the color is too bright. Therefore, the most suitable concentration of BCP in the BCP-(S-PVA) film is 0.025 % w/v.

Fig. 3 The effects of the concentration of BCP and Pen G to the absorption of BCP-(S-PVA) film (λ: 595 nm). Insets is the visual images of color change of BCP-(S-PVA) film in LOAD. (A.) The effects of BCP concentrations when the Pen G concentration was 4.0 ng/mL, (-) is a negative control and (+) is positive control, (B.) Dose response curve of BCP-(S-PVA) film at the concentration of Pen G of 1.0 to 5.0 ng/mL, (C.) Color changed in the LOAD with different BCP concentration (Pen G: 4.0 ng/mL) and (D.) Color changed in the LOAD with different Pen G concentration (BCP: 0.025 % (w/v)).
Note that one of the advantages of the LOAD is that the fat content in the raw milk was separated from the milk, which produce less turbidity of the milk content. Therefore, the color change is much more clearly seen. To obtain the dose response curve, the concentration of Pen G was varied from 1 to 100 ng/mL. When the Pen G concentration has increased from 1 to 100 ng/mL, the absorption value of BCP-(S-PVA) film has increased from 0.403 ± 0.025 to 1.143 ± 0.035. However, there is no significantly change of absorption value when the concentration of Pen G in raw milk is higher than 10 ng/mL. Therefore, the linear range \((R^2: 0.9630)\) of the absorbance value for the detection of Pen G concentration of 1.0 to 5.0 ng/mL in raw milk was considered. The dose response curve in Fig. 3(B) shown that the detection limit of BCP-(S-PVA) film to detect the Pen G in raw milk is 0.17 ng/mL. Therefore, the LOAD with the BCP-(S-PVA) pH indicator is the fast and accurate promising method to determine the residual Penicillin G in raw milk at room temperature.

4. Conclusions

In this research, the concept of the LOAD to detect the residual Pen G in raw milk was proposed. The S-PVA film is suitable used as a polymeric film in the microfluidic channel due to its higher stability and integrity than the PVA film. The PVA concentration in the S-PVA film play an important role to the degree of solubility and the solubility time of the S-PVA film. The integration of BCP pH indicator into the S-PVA film can be used as an indicator to detect the Pen G concentration in raw milk by monitoring the color change of the film. By using a BCP concentration of 0.025 % (w/v), the LOAD with a BCP-(S-PVA) pH indicator can detect the Pen G in raw milk with the detection limit of 0.17 ng/mL. Therefore, the LOAD is fast and accurate alternative method to visualize indicate the concentration of residue Pen G in raw milk.

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