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Suppression of Coffee-ring Effect (CRE) in the Development of Low-cost Diagnostic Kit

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Abstract
One of the applications of membrane technology is using the polymeric membrane as an adsorber or assay-capturing matrix in the diagnostic kits' assembly. This study explores the addition of NaCl into a protein solution to suppress the coffee-ring effect (CRE) in developing a low-cost diagnostic kit. The highest concentration of NaCl addition shows the optimum results with no formation of CRE and high color intensity (low grey scale value). Adding NaCl into the protein solution is a safe and cheap alternative for lowering the cost of assembly, benefiting people in low-resource places.

Keywords: diagnostic-kit; low cost; coffee-ring effect (CRE); NaCl addition

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1.0 Introduction

One of the articles published in the newsroom of the World Health Organization (WHO) in 2023 stated that more than one billion people who live in impoverished communities and limited resources are exposed and affected by neglected tropical infectious diseases: dengue, typhoid, COVID-19, malaria, HIV, tuberculosis and lymphatic filariasis. These diseases cause devastating health and have dire consequences for more than one billion people in terms of quality of life. The term 'neglected' even appears when mentioning those diseases, as they are almost absent from the global health agenda and are almost ignored by global funding agencies.

Besides, most recently, disruption to health services caused by the COVID-19 pandemic has added a further burden. Malaysia is no exception to facing this issue. A study from Aminuddin et al. (2023) and Abdul Rasam et al. (2018) showed the lingering and suggested solutions for controlling dengue and tuberculosis, respectively. Adapting to these issues, the development of diagnostic kits seems a promising choice. It could give quick, sensitive, yet specific results and no longer need to be transported and stored at cold temperatures. These qualities are essential to adapt to the challenges in communities with socio-economic inequalities directly impacting access to healthcare services, adequate housing, electricity, safe water, and sanitation.

One of the techniques in diagnostic kit manufacturing is based on dot-blotting immunoassay, which comprises four essential elements: holder, membrane, protein antigen, or any biological recognition elements, as well as buffer solution (Piazza et al., 2020).

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However, in dot-blotting immunoassay, due to complications extracting such limited samples from biological matter along with the tedious process, it is desired to have a low concentration of antigen during the deposition process on the membrane surface, which is the most crucial process during the assembly of diagnostic kit.

However, during the drying process, the fluid flux towards the edge of the drop is driven by capillary flow, which carries suspended protein antigen to the edge, leading to a ring-like deposit. This effect is undesirable as it can have significant practical implications on the quality of the end products. As such, it is essential to consider the study on the effect of Natrium Chloride (NaCl) addition in protein solution to suppress the coffee-ring effect (CRE). NaCl is an active pharmaceutical ingredient, stabilizer, or excipient. It is well-known for its non-toxic properties, cheap, and readily available (Hani et al., 2019)

In this research, antigen protein has been replaced by lysozyme protein as antigen is not readily available. Therefore, this study was conducted to suppress the CRE by examining the effect of NaCl additive with different concentrations on lysozyme solution. The results were further scanned and analyzed with Image-J software. From the findings, an attempt to offer a formulation strategy from the effect of NaCl is imperative to enable a prediction for CRE suppression by elucidating the mass transfer behavior of un-modified and modified protein solution on the membrane surface.

2.0 Literature Review

Synthetic membranes have gained an important place in daily human life. They are used extensively in various applications, including wastewater treatment, drinking water treatment, gas separation, and biomedical instruments. The most common examples of the application of membranes in the biomedical field are hemodialysis, guided bone regeneration, and diagnostic tool kits. Most membrane researchers in Malaysia and abroad are experts in wastewater treatment and gas separation applications, focusing on new materials and modules for a more efficient and cost-effective separation process. Unfortunately, understanding the roles of membranes, specifically in diagnostic kit applications, remains challenging due to the need for a design methodology and fundamental knowledge.

The dot-blot technique is one of the techniques in developing diagnostic kits, whose mechanism is based on the concept of immunoassay, which is generally classified as a homogenous and heterogeneous assay. In the latter, one protein constituent is immobilized on a solid sorbent surface, while the other components are delivered via the solution phase. A sorbent surface commonly used for protein immobilization in immunodetection is a polymeric membrane (Surti et al., 2022). Nitrocellulose (Shahruddin et al., 2021), nylon (El-Moghazy et al, 2020), and poly (vinylidene fluoride) (PVDF) (Ahmad et al., 2016a) are the most common types of polymeric membrane for immobilization of the protein constituent, due to desirable characteristics such as stable and controllable surface properties, a large surface area, high binding affinity and stability of the biomolecules toward the substrate, simple handling and cheap large-scale production. Each of them has its strengths and weaknesses, in which the final selection of the membrane usually depends on the application of interest.

Typically, the deposited protein or immunoagent on the membrane surface is in a lower concentration. If the working mechanism of the diagnostic kit is based on dot-blotting, a lower concentration of deposited protein or immunoagent often causes an unequal distribution of solute known as the coffee-ring effect (Ahmad et al., 2016b). The coffee-ring effect (CRE) is a ubiquitous phenomenon that commonly occurs in our daily lives. When a liquid is dropped onto flat surfaces, it forms a semi-spherical shape at rest due to external forces, including the gravity pull and surface tension exerted onto the droplet surfaces. The water from the deposited droplet evaporates from the edges known as the three-phase boundary, which is the point where liquid water, the surface, and the air meet. Naturally, when the water starts to evaporate, the other molecules move to take its place. As unevaporated particles start to accumulate at the edge, this leads to the formation of the outer ring. The stain formed due to an uneven evaporation process. This effect is more notable in droplets with low solute concentrations (Okaiyeto et al., 2023).

Thus, it is vital to suppress the coffee-ring effect to avoid misleading diagnostic interpretations. Even though many researchers have worked with the suppression of the coffee-ring effect in a variety of applications, including inkjet printers, DNA microarray printing, and nucleic acid detection, there is a particular lack of detailed investigation in the literature on how to minimize the effect on membrane surface as previous researchers were mainly focused on the ring formation on glass side (Wilkinson et al., 2021; Raghuram et al., 2021), silicon wafer (Yang et al., 2020) and ceramic plate (Shimobayashi et al., 2018) as it provides clear view in the study of the CRE suppression mechanism, without including the effect of solute-substrate (Eg: protein-membrane interaction). Besides, the suppression of CRE has yet to be studied in a biomedical or pharmaceutical context, in which biological or multiple types of solutes are often included. Very often, handling any biological recognition, such as an antigen, requires a delicate process due to its nature, which is more sensitive to changes in the environment.

The ring pattern of CRE from drying droplets was overcome by controlling liquid properties such as surface tension, pH, and surface roughness and altering the temperature of the substrate as well as droplet temperature. Besides that, Marica et al.'s research shows that the addition of nanoparticles affected the final pattern (Marica et al., 2023). The addition of polymer or any other additives is one of the approaches done previously where it can alter the particle deposition of colloidal solution. From all mentioned above, one of the most applicable strategies is the manipulation of the protein concentration via additional solutes, such as chitosan (Wilkinson et al., 2021), polyethylene glycol (PEG) (Shahruddin et al., 2022), NaCl (Gorr et al., 2012) and sucrose (Shimobayashi et al., 2018) in protein's solvent. NaCl seems a promising choice among the additives due to its characteristics, such as non-toxic, cheap, and abundant availability.

Ultimately, developing a commercial diagnostic kit should be considered, considering that it will be used in places with low resources. In short, several diagnostics criteria are sensitive, affordable, specific, user-friendly, rapid and robust, cumbersome equipment-free, and deliverable to end-users. If the target of the diagnostic development is to impact third-world countries, then the research or innovation

needs to be carried out from the client to the experimental design. Thus, a new way for cost minimization must be explored to meet and fulfill one part of the diagnostic kit assembly, specifically for heterogeneous assay.

3.0 Methodology

3.1 Membrane Preparation

The nitrocellulose membranes (HiFlow® Plus Nitrocellulose Membrane, Merck, US) were cut into a dimension of 1cm x 5cm rectangle. The pore size of the nitrocellulose membrane is 0.45 µm.

3.2 Protein Immobilization

In this study, Lysozyme (L-6876, Merck, US) was selected as a model protein. The protein powder was diluted with distilled water to prepare a constant concentration of 0.5 mg/ml. A volume of 1 µL of solution is immobilized on the membrane surface by using a micropipette and left to air dry at room temperature with normal humidity. After drying, the membrane is immersed in 5 mL of Ponceau S dye solution for 5 minutes for signal enhancement. The membrane was then rinsed with distilled water to wash off the undesired membrane background and again air-dried at room temperature. Three different concentrations of NaCl were prepared at 0.01 mg/ml, 0.1 mg/ml, and 2.0 mg/ml. The solution of protein and NaCl was prepared in a 1:1 ratio mixture. A volume of 1 µL of newly-made solution was then spotted on the membrane surface and left to air dry. The steps are repeated for all proteins-NaCl mixtures.

3.3 Dot-blot Analysis

Image acquisition of the dot blot was performed through EPSON L220 3-in-1 (Scanner function) with the following setting: 16>8-bit grayscale and 3200 dpi resolution. Image J (Version Java 1.8) was used for the membrane surface analysis to measure the relative color intensity and the total surface area using the 'line intensity' option (Shahruddin et al., 2022). However, the image analysis by ImageJ provides only relative measurement between the samples for comparison purposes.Figure 1 shows the flow chart for analysis of protein immobilization.

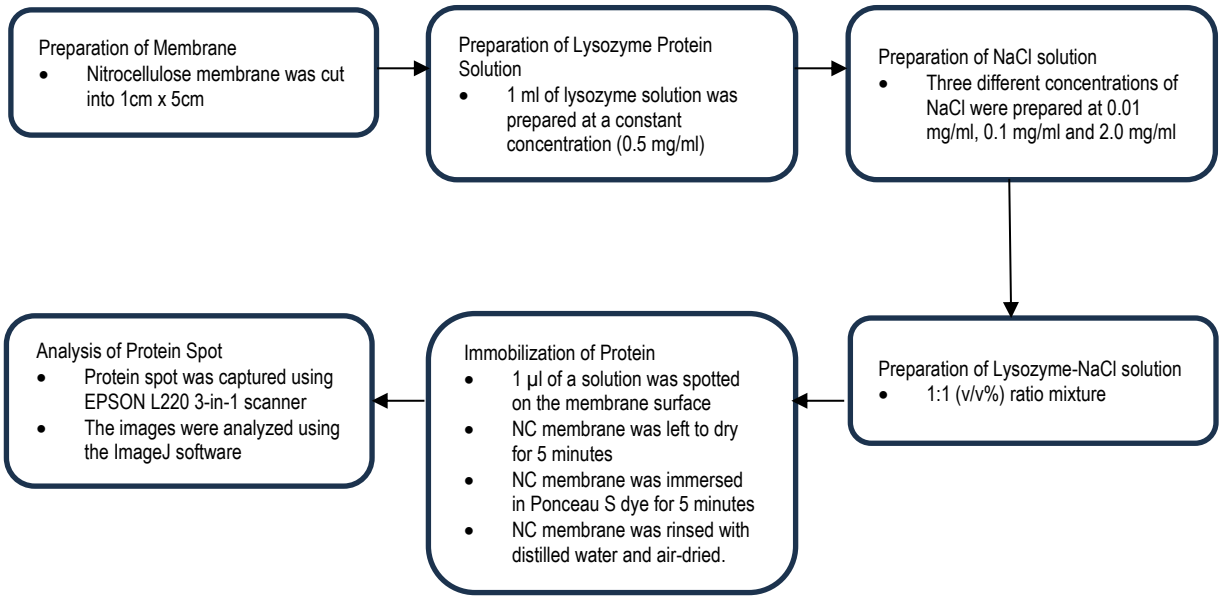

Fig. 1: Flow chart for suppression of coffee-ring effect (CRE) by adding salt (NaCl) to develop a low-cost diagnostic kit

4.0 Results & Discussion

Figure 2 shows the overhead view of the dried protein spot for a blank sample (no addition of NaCl) as a reference and with the addition of NaCl. For evaluating the effect of NaCl, the protein concentration was manipulated from low to high concentrations of NaCl which were 0.01 mg/ml, 0.1 mg/ml, and 2.0 mg/ml. From observation, the blank solution shows a prominent coffee ring at the edges of the deposition, while the solution with a series concentration of NaCl shows a homogenous deposition. It can also be stated that an addition of NaCl, even at low concentration, had a significant effect on the suppression of the coffee ring during the drying process.

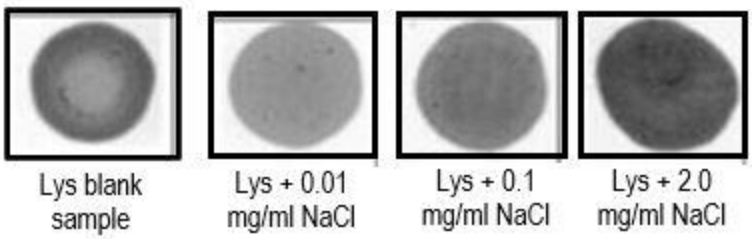

Fig. 2: Image of protein immobilization at 0.5 mg/ml Lys (blank sample) with different NaCl concentration

To overcome the limitations and inconsistency of the image analysis through the observation, the scanned images were further imported into ImageJ to undergo data analysis. Before the analysis process, the image was initially converted into an 8-bit image with black and white condition. The grey value at the y-axis represents the intensity of the dried spot. The greyscale intensity portrays the amount of particles deposition on the membrane surface. The high intensity of dye was expressed in terms of a low reading of grey value.

The graph plotted in Figure 3 demonstrates the differences between the four solutions in the side profile of the dried spot of the solution. For the blank sample, the graph showed two significant peaks at the beginning and the end of the x-axis. This was supported by the image of the spotted membrane (Figure 2: Lys blank sample) where the peaks were at the site of a ring-like pattern that formed at the edge. As mentioned previously, the ring pattern is commonly known as the coffee-ring effect (CRE). Unlike lysozyme protein solution (blank), this phenomenon was observed to be less distinct for the protein mixture with the addition of NaCl. This can be seen in all of the line graphs plotted in Figure 3, where the line graph for the mixture of protein and NaCl displays a more even distribution of intensity throughout the x-axis.

For all samples with the addition of NaCl, there is no obvious peak at the beginning and the end of the x-axis. Comparing the grey value of the lower and higher concentrations of NaCl, a higher concentration (2.00 mg/ml) was preferable as the grey value is lower, approximately around 70, indicating high color intensity. As the concentration became lower, the grey value increased to approximately 110 and 150 for NaCl concentrations of 0.10 mg/ml and 0.01 mg/ml respectively. However, from the interpretation of the line profile, the lowest concentration of NaCl (0.01 mg/ml) shows a more homogenized distribution of protein particles, reflected from the minimum jagged line along the x-axis. The particles were more evenly distributed as the y-axis was in consistent value. From Figure 3., the jagged line of samples with the highest concentration of NaCl (2.00 mg/ml), from end to end is between the grey value of 70 – 100, whereas for the 0.10 mg/ml NaCl concentration, the grey value from end to end is approximately around 110 – 140. Adding NaCl at 0.01 mg/ml shows the most homogenized distribution, with the grey value consistently at ~150. This sample also shows the maximum CRE suppression with the highest peak observed at the center and becomes lower towards the edge of the protein spot.

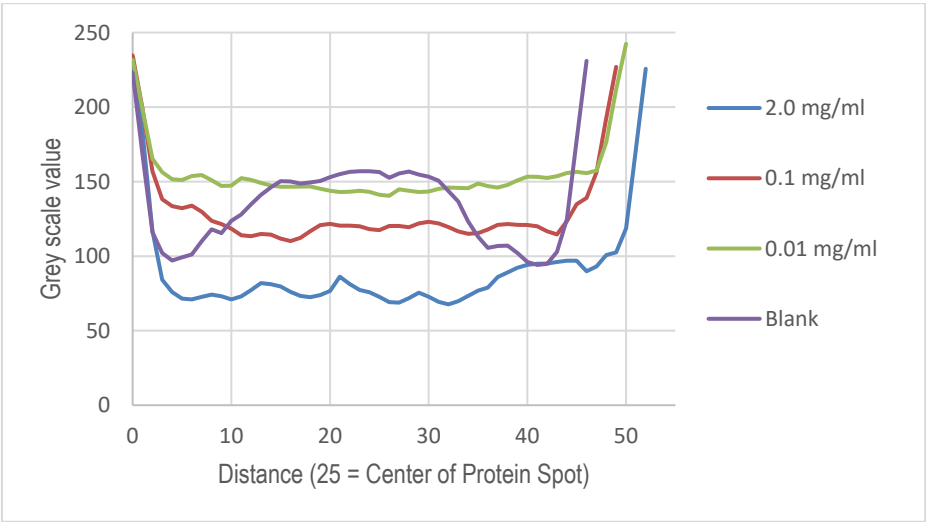

Fig. 3: Greyscale value of control (blank solution) with individual mixture solution at different NaCl concentration

From the interpretation of the results, it is clear that the addition of NaCl significantly affects the mass transfer behavior of the protein solute during the evaporation process. The addition of NaCl will increase the crowding effect in the protein solution and act as a viscous body, causing the radial flow of the protein solution from the center, inhibited. In a blank solution, the mass transfer of protein solute from the center of the droplet to the edge was faster, caused by the capillary flow. In the other NaCl-containing solutions, an increased viscosity provided resistance to the movement of solvent and solutes on the membrane surface (Wilkinson et al., 2021).

Besides the crowding effect, the forces and interactions acting on protein molecules during the evaporation are included. Four major forces and interactions are capillary flow, Marangoni flow, the interaction between the protein and membrane, and the interaction between the protein molecules (Yang et al., 2022). At pH 7, the positively charged lysozyme molecules adsorb to the negatively charged nitrocellulose membrane due to protein-substrate interaction. For low concentrations of protein solute, the capillary flow is more prominent, causing the mass transfer of protein to the periphery and eventually forming the ring. However, as part of the protein molecules are transported to the edge of the droplet, the ionic strength of the remaining solution increases and affects the protein-protein interaction. Clusters of lysozymes have been known to form in such cases. Thus, the addition of NaCl caused the formation of clusters as the droplets evaporated. With an increase in NaCl concentration, clusters are formed and hinder the mass transfer of protein to the periphery by force of capillary and suppress the formation of CRE (Gorr et al., 2013). Add two additional references

5.0 Conclusions & Recommendations

In this study, it was concluded that the development of a low-cost diagnostic kit is important in overcoming the issues of neglected tropical infectious diseases as it is targeted to be used in low-resource places. The addition of NaCl into the protein solution will contribute to the development of a low-cost diagnostic kit by suppressing the formation of CRE which might cause a misleading interpretation of the diagnostic. From the results, it can be concluded that an addition of NaCl, even at low concentration, had a significant effect on the suppression of coffee-ring during the drying process. The highest concentration of NaCl addition (2.00 mg/ml) shows the optimum results with no formation of CRE and high protein color intensity (low grey scale value). However, from the interpretation of the line profile, the lowest concentration of NaCl (0.01 mg/ml) shows a more homogenized distribution of protein particles, reflected from the minimum jagged line along the x-axis. Adding NaCl into the protein solution should be considered a safe and cheap alternative for lowering the cost of diagnostic kit assembly. For future research, it is recommended to replace the use of lysozyme solution as a model protein with the real antigen or any biological recognition elements, harvested from the microorganisms for verification and biocompatibility test. It is expected that the knowledge developed from this study will provide some insights in the development of low-cost diagnostic kit.

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Paper Contribution to Related Field of Study

This research paper aims to strengthen the medical and healthcare services in low-resource places without electricity and clean water supply, minimal or nonexistent infrastructure, and lack of well-trained personnel. This is also in line with one of the Sustainable Development Goal (SDG) elements, which is to ensure the excellent health and well-being of the community in developed or under-developed countries or in what is known as the forgotten bottom billion. In Malaysia's context, the community's health and the quality of healthcare will continue to be improved by acknowledging that the government still needs to address the shortage of medical equipment and specialists, especially in rural areas.

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