CHAPTER 3 METHODOLOGY

This chapter presents the methodology to accomplish the objectives of the project which consists of reagents, materials, and equipment that were used in this work. In addition, chemical preparation, design and fabrication of a test strip, urine sample collection, interpretation of results on the test strip, comparison with previous work are also described.

3.1 Reagents and materials

1. Sample pad (Millipore C048)
2. Conjugate pad (Ahlstrom 8964)
3. Nitrocellulosemembrane (Unisart CN 140)
4. Absorbent pad (Whatman 470)
5. Backing card (Polyethylene sheet)
6. Carbon nanoparticles (Spezial Schwartz 4)
7. Deionized water
8. Hydrochloric (HCl)
9. Sodium hydroxide (NaOH)
10. Bovine serum albumin (BSA)
11. Goat anti-mouseIgG
12. Primary anti-NGAL antibody
13. Secondary anti-NGAL antibody
14. Recombinant NGAL protein
15. Borate buffer (BB)
16. Phosphate-buffered saline (PBS)
17. Boric acid (H_3BO_3)
18. Sodium tetraborate ($Na_2B_4O_7.10 H_2O$)
19. Washing buffer (WB)
20. Storage buffer (SB)
21. Sodium azide (NaN_3)
22. Sucrose
23. Trehalose
24. Dimethyl formamide
25. Hexane
26. Urine sample from healthy volunteers
27. Methanol
28. Micro BCA^{TM} Protein Assay Kit
29. Silica gel

3.2 Equipment

1. pH meter
2. Beaker
3. Water bath
4. Stirring Rod
5. Centrifugal tubes
6. Centrifuge
7. Pipette
8. Dropper
9. Micropipette
10. Micropipette tips
11. Oven
12. Forceps
13. Desiccated chamber
14. Lancet
15. Spatula
16. Glove
17. Scissors
18. Surgical blade No.11
19. Scalpel handles No.3
20. Microcentrifuge
21. Microcentrifuge tubes
22. Ultrasonic Bath
23. Spectrophotometer
24. Automatic cutter (Matrix™ 2360 Programmable Shear)
25. Automatic sprayer (IsoFlow™ Dispenser)
26. Shaker
27. Sealer
28. Cassettes
29. Scanner (CanoScan LiED 700F)
30. Foil pouche

3.3 Methods

3.3.1 Preparation of carbon nanoparticle

1. The soot from burning toluene was condensed on a glass surface and then collected.
2. Carbon was suspended in tenfold the amount by weight of toluene, and boiled in a flask with a backflow condenser for 30 min, then cooled and filtered through a Schott's glass filter.
3. The same procedure but without heating and cooling was repeated five times and resulted in a completely discolored solvent after the last filtration.
4. The carbon was washed at room temperature with dimethyl formamide and hexane five more times.
5. The final product was dried under vacuum to ensure removal of traces of organic solvent.
6. The carbon was annealed at 250 °C for 12 hours to achieve a fixed weight. A matt-black dust like hydrophobic powder was obtained.

3.3.2 Preparation of buffer

1. Borate buffer (BB): 100 mM, pH 8.8, was prepared by mixing 100 mM solutions of H_3BO_3 and $Na_2B_4O_7 \cdot 10\ H_2O$ in ultrapure water.
2. BB (5 mM) was obtained by diluting 100 mM of BB appropriately.
3. Several buffers were prepared from brovine serum albumin (BSA) and tween 20 which are,
   - Washing buffer (WB, 5 mM BB, 1% (w/v) BSA)
   - Storage buffer (SB, 100 mM BB, 1% (w/v) BSA)

   And NaN3 was added to all buffers to a final concentration of 0.02%(w/v).

3.3.3 Preparation of antibody-carbon nanoparticle conjugate

1. 10 mg of carbon was suspended in 1 ml of deionized water and sonicated for 5 min and then a colloidal carbon suspension was prepared as a stock at 1% (w/v).
2. The resulting 1% (w/v) carbon suspension was fivefold diluted in 5 mM BB and sonicated for 5 min to give a concentration of 0.2% (w/v)
3. Next, 350µg/mL of anti-NGAL antibody in the diluted carbon suspension was prepared by mixing 350µg anti-NGAL antibody with 1 mL of the diluted carbon suspension and then shaker for 3 hours at 150 rpm and room temperature.
4. Then, this suspension was centrifuged at 11,000 rpm for 15 min. Next time, the supernatant was removed and the pellet is washed with 1 mL of WB to remove unbound protein.
5. 1 mL of WB was added to the pellet and the mixture was centrifuged at 11,000 rpm for 15 min and repeat in step 4 for 2 times.
6. After the final wash, the pellet was resuspended in 1 mL of SB and store at 4°C until use.
7. 10% of sucrose and 5% of trehalose were added to the antibody-carbon conjugate and then mixed with the sample before test on test strip.

3.3.4 Dot blot test

1. The concentration of NGAL protein in PBS is varied ranging from 50-1000 ng/mL
2. Each concentration is spotted on nitrocellulose membrane by slowly pipetting 2 µL/spot onto the different locations of the membrane.
3. The remaining non-specific sites are blocked by submersing the membrane into a solution 5%(w/v) BSA in 0.1 M phosphate buffer at pH7 and drying at 37°C 1 hour.
4. The NGAL protein membrane is submerged into antibody-carbon nanoparticle conjugate volume 10 µL with a concentration of 1% (w/v) solids in 0.01 M PBS and incubated at room temperature for 30 min.
5. The membrane is wash with 0.01M PBS, air-dried and scanned.

3.3.5 Analysis of the urine samples using the dot blot method

- An image of the dot spots was scanned using a scanner. The color intensity of the dot spots was analyzed by image analysis.

3.4 The results

The results are divided into 2categories which are positive, negative, as shown in Figure 3.1.

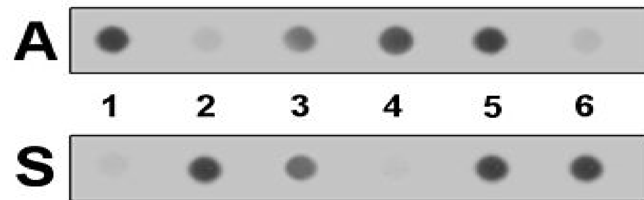

Figure 3.1 The results of dot blot method

- **Positive :** If the color appears.
- **Negative :** If the color does not show.