CHAPTER 4 RESULTS AND DISCUSSION

This chapter consists of experimental results and discussion. First, the size of carbon was analyzed. Second, the preparation of the antibody-carbon nanoparticle conjugate was determined. The last part is to test the detection of NGAL protein in phosphate buffer saline (PBS) as a sample on Nitrocellulose membrane by the lateral flow test and dot blot method.

4.1 The size of antibody-carbon nanoparticle

When carbon conjugate was kept at 4°C for a long time, it tended to aggregate to a large particle. The size of the carbon conjugate should be known to find the suitable size. The carbon conjugate was then prepared at different storage time after sonication (day 0, 1, 3, 7, 15, 30 and 60) for a size analysis. The results can be summarized by plotting the trend of size of carbon conjugate after sonication as shown in Figure 4.1.

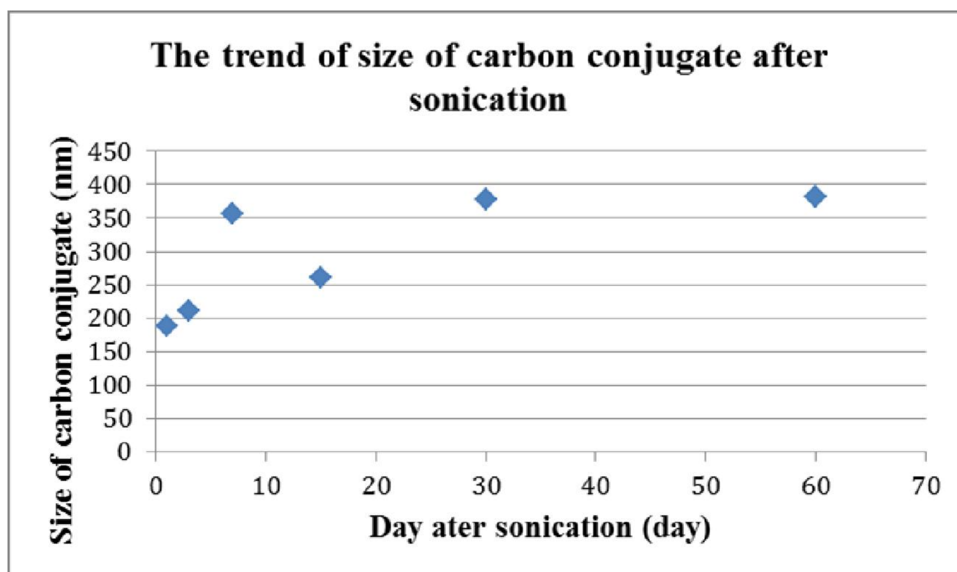


Figure 4.1 The trend of size of carbon conjugate after sonication.

From the table and graph, the size of carbon conjugate tended to be larger when it was kept for a longer time. However, the particle size 30 days after sonication and 60 days after sonication were quite similar sizes which were 378.43 and 382.07 nm, respectively. Therefore, the storage time after sonication only affected the aggregate size of the carbon at earlier time (up to 30 days in storage), but it hardly affected the carbon size when it was kept for a long time, which was 60 days after sonication. Moreover, the aggregate size of carbon does not affect this system because 10 µm pore size of nitrocellulose membrane [26] was larger than the aggregate size of carbon.

4.2 Preparation of the antibody-carbon nanoparticle conjugate and the results of each preparation

4.2.1 Antibody: Monoclonal anti-NGAL antibody (P5E1) from BBI solution

4.2.1.1 Varying 0.2% (w/v) carbon suspension in antibody-carbon nanoparticle conjugate

The antibody-carbon nanoparticle conjugate was prepared using 350 µg/ml of anti-NGAL antibody. Anti-NGAL antibody was mixed with the 0.2% (w/v) carbon suspension for 3 hours at room temperature. The antibody-carbon conjugate was centrifuged at 11,000 rpm for 15 minutes and then the supernatant and unbound protein was removed by adding washing buffer (WB). Its volume was adjusted by adding 1 ml storage buffer (SB) and stored at 4°C until use, as shown in Figure 4.2.

Figure 4.2 Varying 0.2% (w/v) carbon suspension in antibody-carbon nanoparticle conjugate.

Then the amount of anti-NGAL antibody in the 0.2% (w/v) carbon suspension was determined from the BSA standard curve, as shown in Figure 4.3. The conjugation efficiency was 89.23%, as shown in Table 4.1. The conjugation efficiency was lower due to higher initial antibody loading is shown in Table 4.1.

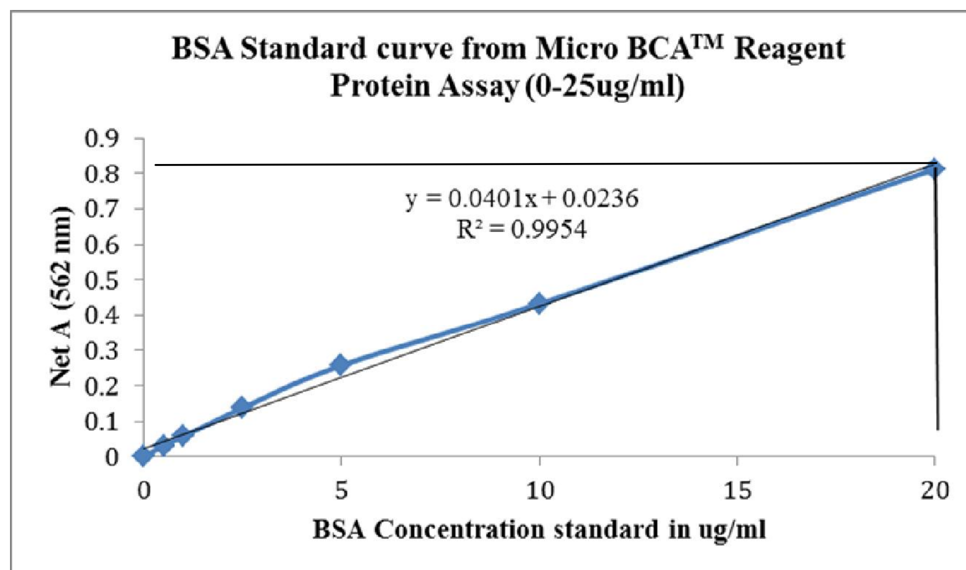


Figure 4.3 BSA standard curve from Micro BCA™ Reagent Protein Assay (0 – 20 µg/ml).

Table 4.1 The percent of carbon conjugate at 350 µg/ml in 0.2% (w/v) carbon suspension of anti-NGAL antibody concentration

Concentration (µg/ml)	Initial concentration (µg/ml)	Free concentration (µg/ml)	% Conjugation efficiency
350	350	37.71	89.23

4.2.1.1.1 Test the detection of protein (2 µL/spot) on Nitrocellulose membrane by a lateral flow test

The concentrations of NGAL protein in PBS were varied from 50, 100, 500 and 1000 ng/ml and spotted by slowly pipetting 2 µL/spot onto different locations of the nitrocellulose membrane. The remaining non-specific sites were blocked by immersing the membrane into a solution containing 5% (w/v) BSA in 0.1 M PBS at pH 7 and dried at 37°C for 1 hour. After that, the membrane was partially dipped into 0.2% (w/v) antibody-carbon nanoparticle conjugate solution and incubated at room temperature for 30 minutes. The membrane was washed with PBS and air-dried, as shown in Figure 4.4.

Figure 4.4 Flow of 0.2% (w/v) antibody-carbon nanoparticle conjugate with a concentration on the membrane previously spotted with various concentrations of NGAL protein at 2 µL/spot.

The antibody-carbon conjugate solution flowed through the membrane without any problem, although black stain was present due to excess amount of carbon conjugate. However, no black spot was observed. It was possible that the color intensity was not sufficiently strong to be analyzed by naked eyes. One explanation is that pipetting 2 µL/spot onto the membrane caused a large spot because when the protein was dropped on the membrane, their spots were combined together. Then, the protein spot would be reduced to 1 µL/spot for the next study.

4.2.1.1.2 Test the detection of protein (1µL/spot) on Nitrocellulose membrane by lateral flow test

The concentrations of NGAL protein in PBS were varied 50, 100, 500 and 1000 ng/ml and each concentration was spotted by slowly pipetting 1 µL/spot onto different locations of the nitrocellulose membrane. The protocol for the detection of protein was the same as in the previous study. The result is shown in Figure 4.5.

Figure 4.5 Flow of 0.2% (w/v) antibody-carbon nanoparticle conjugate on the membrane where various concentrations of NGAL protein were spotted at 1 µL/spot.

The result of this study could not indicate the presence of the protein, which was spotted on the membrane. The antibody-carbon nanoparticle conjugate could not detect the protein by the lateral flow immunoassay system. Then, the direction addition of the conjugate solution would be used in the next study.

4.2.1.1.3 Test the detection of protein (1µL/spot) on Nitrocellulose membrane by dot blot method

The concentrations of NGAL protein in PBS were varied from 50, 100, 500 and 1000 ng/ml and each concentration was spotted by slowly pipetting 1 µL/spot onto the different locations of the nitrocellulose membrane. The remaining non-specific site was blocked by submersing into a solution 5% (w/v) BSA in 0.1 M PBS at pH 7 and dried at 37°C for 1 hour. After that, the antibody-conjugate solution was directed added to the spots by slowly pipetting 1 µL/spot of carbon and incubated at room temperature for 30 min. The membrane was washed with PBS and air-dried, as shown in Figure 4.6.

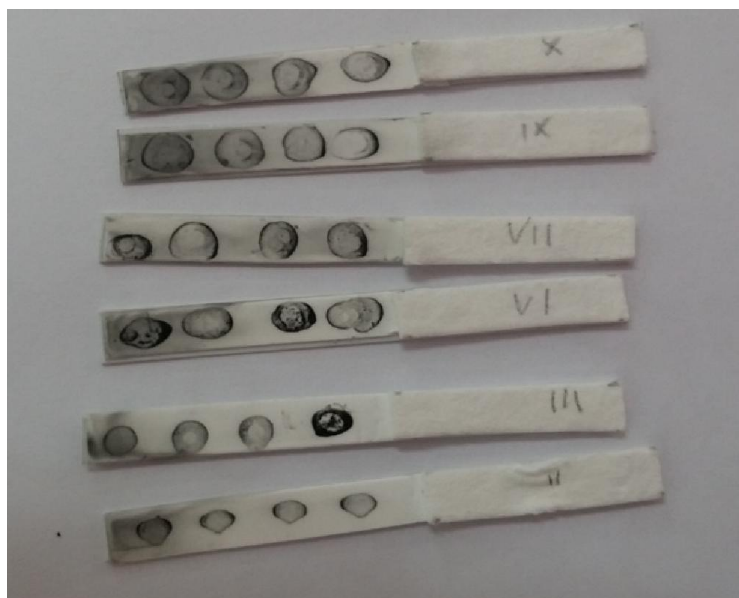


Figure 4.6 Dot blot of antibody-carbon nanoparticle conjugate at a concentration 0.2% (w/v) carbon suspension on the membrane with 1 µL/spot of each concentration of protein.

The results of this study could not indicate the presence of the protein, which was spotted on the membrane. In the washing step of carbon using PBS, which excess carbon would be normally removed from the membrane, the spots of carbon still stuck on the membrane. Thus, the antibody-carbon nanoparticle conjugate could not be used to detect the protein by the dot blot method. Maybe the concentration of the carbon suspension was not sufficient or the step of washing was not good enough; therefore, increasing the concentration of the carbon suspension and improving the washing step would be done in the next study.

4.2.1.2 Varying 1.0% and 2.0% (w/v) carbon suspension in antibody-carbon nanoparticle conjugate

4.2.1.2.1 Antibody-carbon nanoparticle conjugate were prepared by centrifuging 0.2% (w/v) carbon suspension in antibody-carbon nanoparticle conjugate

The antibody-carbon nanoparticle conjugate was prepared using 0.2% (w/v) carbon suspension in antibody-carbon nanoparticle conjugate, which is a carbon stock. The carbon stock was centrifuged at 11,000 rpm for 15 minutes and then the supernatant was removed. After that, the storage buffer (SB) was added to make the concentration of carbon suspension to 1.0% and 2.0% (w/v), respectively and stored at 4°C until use.

The percent carbon conjugate of those antibody-carbon nanoparticle conjugates could not be determined because the color intensities of them were higher than the color intensity of the BSA standard curve, as shown in Figure 4.7. Therefore, centrifuge is not recommended for this study because protein might be removed from the conjugate and the percent conjugation efficiency must be decreased.

Figure 4.7 The color intensity of the highest concentration of BSA standard curve (40 μg/ml) and sample.

4.2.1.2.2 Antibody-carbon nanoparticle conjugate were prepared by a new preparation method

The antibody-carbon nanoparticle conjugate was prepared using 350 μg/ml of anti-NGAL antibody. Anti-NGAL antibody was mixed with the 1.0% and 2.0% (w/v) carbon suspension, respectively. The protocol for the preparation of antibody carbon nanoparticle conjugate was the same as described previously. Then, the volume was adjusted by adding 1 mL storage buffer (SB) and the solution was stored at 4°C until use, as shown in Figure 4.8.

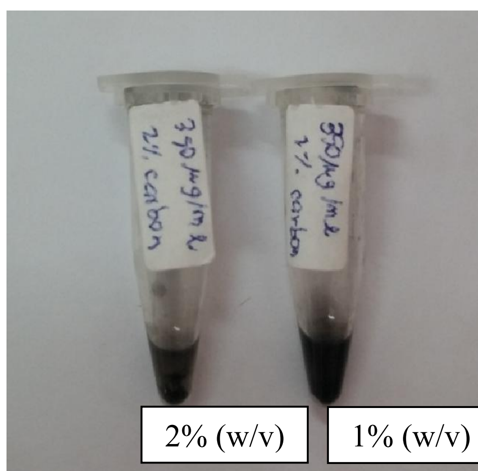


Figure 4.8 Varying 1.0% (right) and 2.0% (left) (w/v) carbon suspension in antibody-carbon nanoparticle conjugate.

The amount of anti-NGAL antibody in the 1.0% and 2.0% (w/v) carbon suspension were determined from the BSA standard curve, as shown in Figure 4.9. The percent carbon conjugate of this antibody-carbon nanoparticle conjugate was 53.75% and 8.34%, respectively, as shown in Table 4.2. The conjugation efficiency was extremely lower especially in 2.0% (w/v) carbon suspension due to higher initial antibody loading. Because much lower volume was used in this preparation, it may have caused incomplete mixing, leading to poor binding between antibody and carbon nanoparticles and eventually lower conjugation efficiency. However, these carbon stocks would be used in the next study.

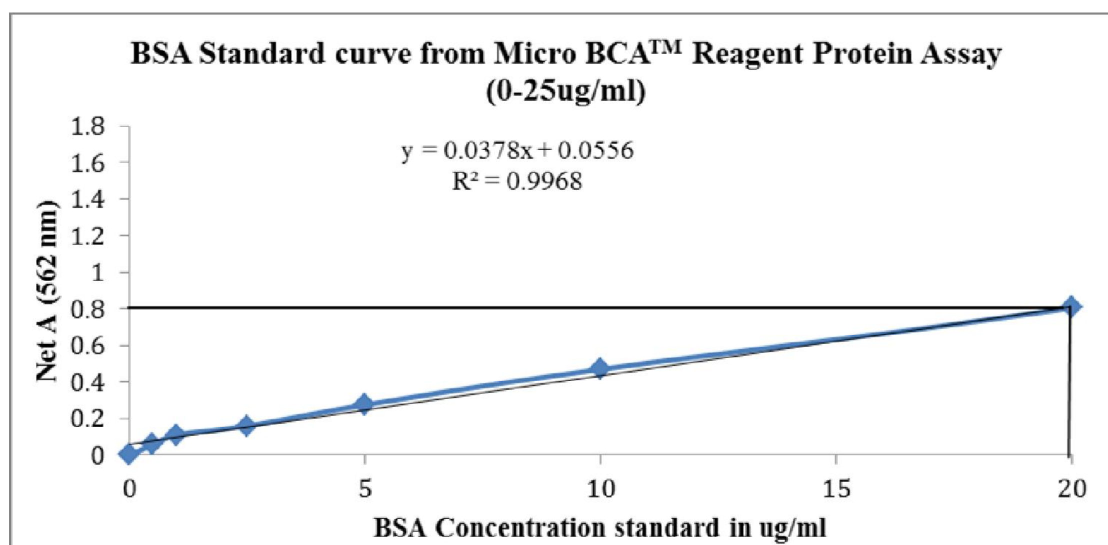


Figure 4.9 BSA standard curve from Micro BCA™ Reagent Protein Assay (0-20μg/ml)

Table 4.2 The percent of carbon conjugate at 350 µg/ml in 1.0% and 2.0% (w/v) carbon suspension of anti-NGAL antibody concentration

% Carbon suspension	Concentration (µg/ml)	Initial concentration (µg/ml)	Free concentration (µg/ml)	% Carbon conjugate
1.0	350	350	161.87	53.75
2.0	350	350	320.79	8.34

4.2.1.2.2.1 Test the detection of protein (1µL/spot) on Nitrocellulose membrane by lateral flow test

The concentrations of NGAL protein in PBS were varied from 50, 100, 500 and 1000 ng/ml and each concentration was spotted by slowly pipetting 1 µL/spot onto the different locations of the nitrocellulose membrane. The protocol for the detection of protein was the same as in the previous study. The results are shown in Figure 4.9

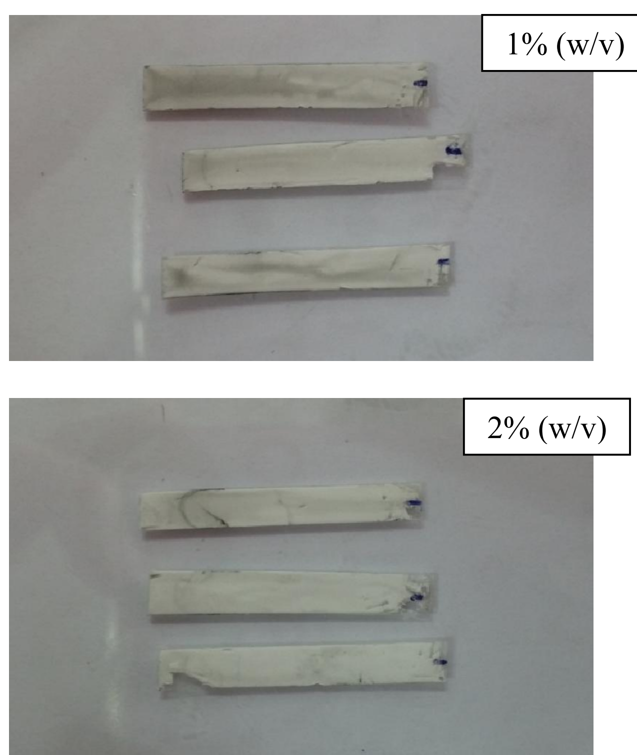


Figure 4.10 Flow of antibody-carbon nanoparticle conjugate with a concentration 1.0 % and 2.0% (w/v) carbon suspension on the membrane with 1 µL/spot of each concentration of protein.

The result of this study could not indicate the presence of the protein, which was spotted on the membrane. Maybe the percent of carbon conjugated was too low, the protein and antibody could not bind together.

4.2.2 Antibody: Human Lipocalin-2/NGAL Biotinylated (BAF1757) from R&D system

4.2.2.1 Label: carbon nanoparticle conjugate

4.2.2.1.1 Varying the ratio of carbon suspension and antibody at 5000:1

In this study, the antibody-carbon nanoparticle conjugate was prepared using 0.4 µg/ml of anti-NGAL antibody. Anti-NGAL antibody was mixed with the 0.2% (w/v) carbon suspension. Due to low concentration of the antibody, the concentration ratio between carbon suspension and antibody would be used instead, which is 5000:1. The speed of the shaker was set at 150 and 200 rpm. The antibody-carbon nanoparticle conjugate at 150 and 200 rpm were called CARBON1 and CARBON2, respectively. The protocol for the preparation of the antibody carbon nanoparticle conjugate was the same as previously. Then, the solution was stored at 4°C until use, as shown in Figure 4.11.

Figure 4.11 Varying the ratio of carbon suspension and antibody at 5000:1 in using different stirring speeds: 150 rpm (left) and 200 rpm (right).

The percent carbon conjugate of those antibody-carbon nanoparticle conjugates could not be determined because the volume of the carbon conjugate is too low.

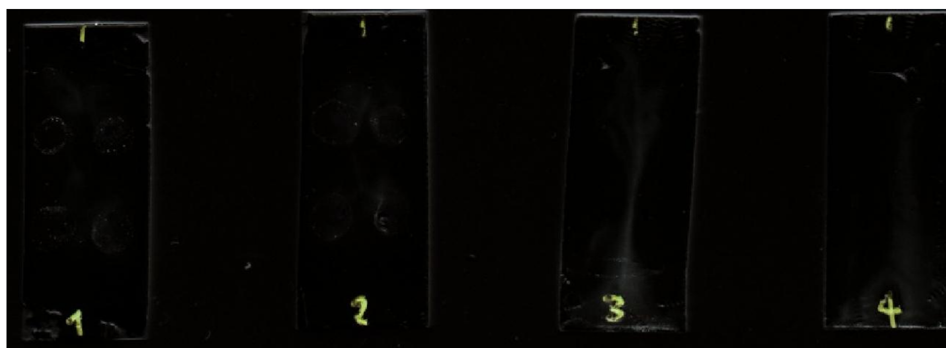
4.2.2.1.1.1 Test the detection of protein on Nitrocellulose membrane by dot blot and lateral flow

The experiment of this study consists of 3 sets. First, CARBON1 and CARBON2 were used to study the effect of the shaker's speed. Second, the efficiency of CARBON1 was determined by the dot blot and lateral flow methods. The last part, the efficiency of CARBON2 was determined by the dot blot and lateral flow methods similar to the second study using CARBON1.

The protocol for the detection of protein was the same as in the previous study. The size of membrane was 25mm x 10mm that was larger than previously. The reason to use this

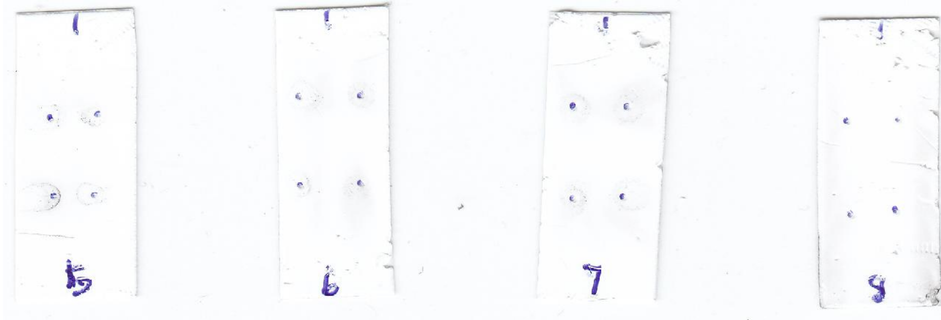
membrane size is to avoid the combination of each spot. The concentrations of NGAL protein in PBS were varied 50, 100, 500 and 1000 ng/ml and each concentration was spotted by slowly pipetting 2 µL/spot onto the different locations of the nitrocellulose membrane. After that, the membrane was blocked with 5% (w/v) BSA by immersing and dried for 1 hour, then the carbon conjugate was dropped on the surface for the dot blot, and membrane was dipped into the carbon conjugate solution for the lateral flow test. After the membrane was incubated for 30 min, the membrane was washed with PBS and air-dried. The results are shown in Figure 4.12-4.14 and Table 4.3.

a) 1. CARBON1& dot blot, 2. CARBON2 & dot blot, 3. CARBON1 & lateral flow, 4. CARBON2 & lateral flow.

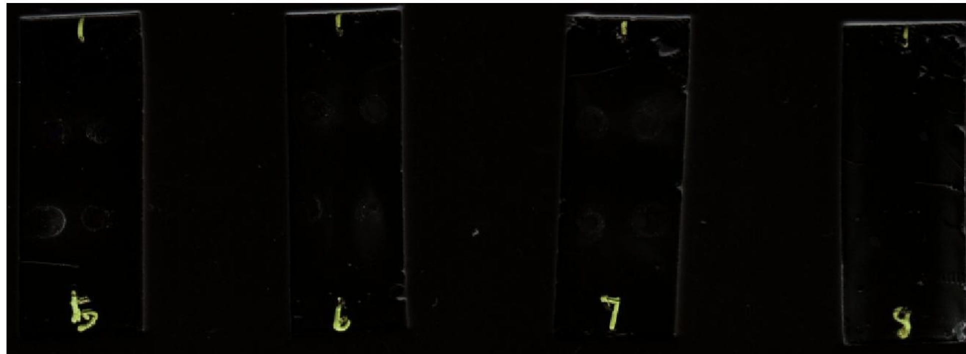


b) 1. CARBON1& dot blot, 2. CARBON2 & dot blot, 3. CARBON1 & lateral flow, 4. CARBON2 & lateral flow.

Figure 4.12 The first set of the experiment: a) after scanning b) after scanning and inverting.



a) 5.-7. CARBON1& dot blot, 8. CARBON1 & lateral flow.



b) 5.-7. CARBON1& dot blot, 8. CARBON1 & lateral flow.

Figure 4.13 The second set of the experiment: a) after scanning b) after scanning and inverting.

a) 9.-11. CARBON2& dot blot, 12. CARBON2 & lateral flow.

b) 9.-11. CARBON2& dot blot, 12. CARBON2 & lateral flow.

Figure 4.14 The third set of the experiment: a) after scanning b) after scanning and inverting.

Table 4.3 The intensities of each spot of CARBON1 and CARBON3

Sample	Intensity of protein 50 ng/ml	Intensity of protein 100 ng/ml	Intensity of protein 500 ng/ml	Intensity of protein 1000 ng/ml
CARBON 1	5.19	7.29	6.61	6.33
CARBON 2	6.73	6.31	7.06	5.60

When the speeds of the shaker were varied to 150 and 200 rpm to study the effect of speed, both results were not in dot blot and in lateral flow. Therefore, higher speed of the shaker did not conjugate the carbon particles and antibody any better. Then, 150 rpm of speed would be used in the next experiment. But the color intensity of these three sets was still too pale and the carbon stain still stuck on the membrane. Thus, the concentration ratio of carbon and antibody should be reduced.

4.2.2.1.2 Varying the ratio of carbon suspension and antibody at 2000:1 and 667:1

In this study, the antibody-carbon nanoparticle conjugate was prepared using 1.0 and 3.0 µg/ml of anti-NGAL antibody. Anti-NGAL antibody was mixed with the 0.2% (w/v) carbon suspension. This solution has a very low concentration of antibody. The concentration ration between carbon suspension and antibody is 2000:1 and 667:1 which were called CARBON3 and CARBON4, respectively. The speed of the shaker was at 150 rpm. The protocol for the preparation of antibody carbon nanoparticle conjugate was the same as described previously. Then, the solution was stored at 4°C until use, as shown in Figure 4.15.

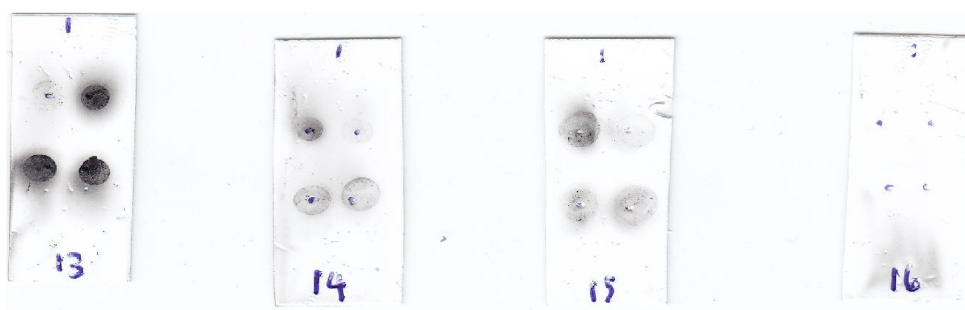
Figure 4.15 Varying the ratio of carbon suspension and antibody at 2000:1 (left) and 667: 1 (right).

The percent carbon conjugate to those antibody-carbon nanoparticle conjugates could not be determined because the volume of the carbon conjugate was too low.

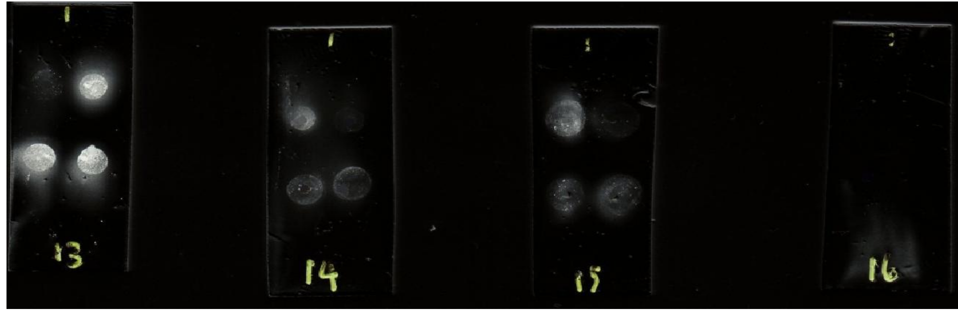
4.2.2.1.2.1 Test the detection of protein on Nitrocellulose membrane by dot blot and lateral flow

The experiment of this study consists of 3 sets which were CARBON1, CARBON3 and CARBON4. The efficiencies of the carbon conjugate solutions were determined by the dot blot and lateral flow methods. Finally, the result of each set was compared. For CARBON1, the membrane number 13, 14 and 15 were used to test by the dot blot method and the membrane number 16 and 17 were used to test by the lateral flow method. In the same way, CARBON3 and CARBON4 were also tested like CARBON1. For CARBON2, the membrane number 18, 19 and 20 were used to test by the dot blot and the membrane number 21 and 22 were used to test by the lateral flow. For CARBON3, the membrane number 23, 24 and 25 were used to test by the dot blot and the membrane number 26 and 27 were used to test by the lateral flow.

The protocol for the detection of protein was the same as described previously. The results are shown in Figure 4.16-4.19 and Table 4.4

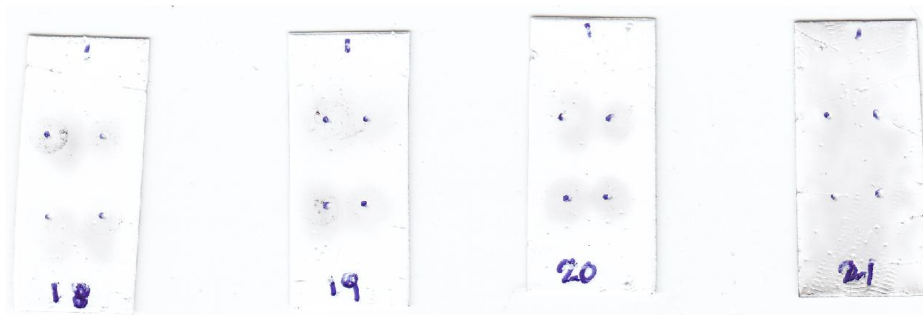


a) 13.-15. CARBON1& dot blot, 16. CARBON1 & lateral flow.

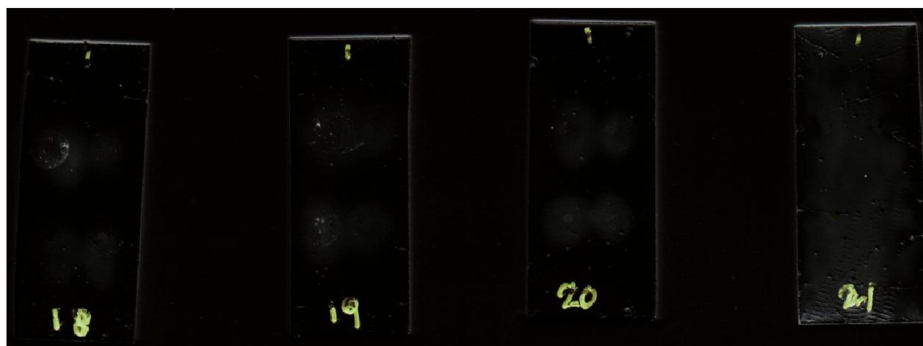


b) 13.-15. CARBON1& dot blot, 16. CARBON1 & lateral flow.

Figure 4.16 The first set of the experiment: a) after scanning b) after scanning and inverting.



a) 18.-20. CARBON3& dot blot, 21. CARBON3 & lateral flow.



b) 18.-20. CARBON3& dot blot, 21. CARBON3& lateral flow.

Figure 4.17 The second set of the experiment: a) after scanning b) after scanning and inverting.

a) 23.-25. CARBON4& dot blot.

b) 23.-25. CARBON4& dot blot.

Figure 4.18 The third set of the experiment: a) after scanning b) after scanning and inverting.

Figure 4.19 The failure of lateral flow on the membrane: 17. CARBON1, 22. CARBON3, 26. and 27. CARBON4.

Table 4.4 The intensities of each spot of CARBON1, CARBON3 and CARBON4

Sample	Intensity of protein 50 ng/ml	Intensity of protein 100 ng/ml	Intensity of protein 500 ng/ml	Intensity of protein 1000 ng/ml
CARBON 1	81.78	14.67	43.27	37.28
CARBON 3	13.02	8.97	11.72	10.26
CARBON 4	7.22	6.28	9.92	9.78

When the concentration ratios were varied from 5000:1, 2000:1 to 667:1 to study the effect of the concentration ratio. The results of CARBON1, which had the highest concentration ratio, had high intensity of each spot when compared with others. Because of the highest concentration ratio, the carbon stain on the membrane still appeared. The intensity of CARBON3 was higher than CARBON4. But for CARBON3 and CARBON4, the carbon stain on the membrane hardly appeared and their carbon stain were less than the carbon stain of CARBON1. Therefore, the higher concentration ratio caused more none specific binding. The antibody was not captured at the protein spots well, and the color intensity was too pale.

From the results of CARBON3 and CARBON4, when the concentration ratio was reduced, the color intensity of each spot was not significantly different. Thus, reducing the concentration ratio between carbon and antibody did not show noticable changes in intensity values. The concentration ratio between carbon and antibody at 2000:1 would be used in the next study.

On the other hand, when lateral flow was used to detect the protien, it cannot easily flow through the membrane. Because of the size of the membrane were too large, the capillary force of nitrocelloluse membrane was 140 sec/4cm [26]. Moreover, this test used a lot of carbon conjugate; therefore, too much carbon particles might be stuck inside the membrane pore that the carbon conjugate could not flow through the membrane. When compared to the first prototype lateral flow test strip, only 1.5 µl of the carbon conjugate and 100 µl of samples were used [27]. But in this test, 2 µl of the sample was dropped on the membrane and 20 µl of the carbon conjugate was used to flow through the membrane.

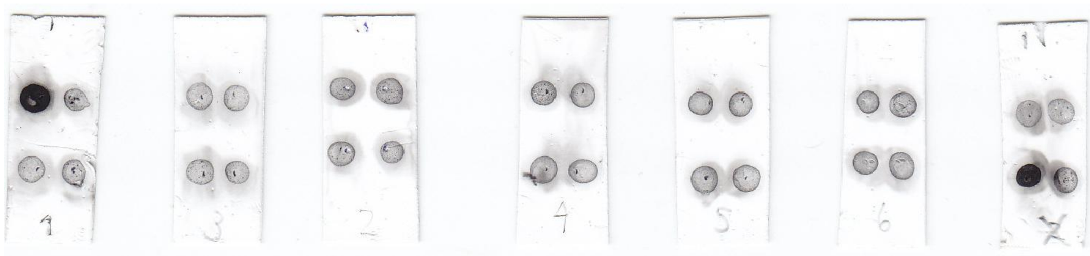
4.2.2.1.3 Repeating the ratio of carbon suspension and antibody at 2000:1 by dot blot

After comparing with three concentration ratios, 2000:1 was selected to test in this study. In this study, the new solution with concentration ratio 2000:1, which was called CARBON5, was prepared to compare with CARBON3. The preparation of antibody-carbon nanoparticle conjugate was the same as described previously, as shown in Figure 4.20.

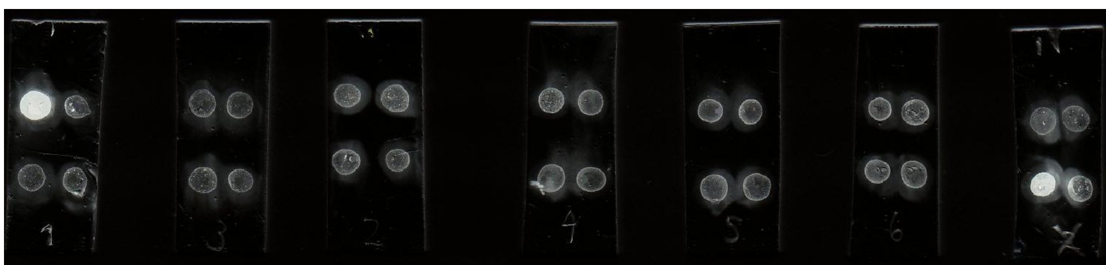
Figure 4.20 Repeating the ratio of carbon suspension and antibody to 2000:1.

4.2.2.1.3.1 Test the detection of protein on Nitrocellulose membrane by dot blot and lateral flow

First, the efficiency of CARBON3 and CARBON 5 were determined on the membrane size 25mm x10mm by the dot blot method. In this experiment, both CARBON3 and CARBON5 were tested by two alternatives. The first alternative was to spot the protein and the carbon conjugate at 2 µl/spot, 1spot. The other was to spot the protein and the carbon conjugate at 1 µl/spot twice to reduce the size of the spots from excessive dispersion. The results are shown in Figure 4.21-4.22 and Table 4.5



a) 2 µl/spot, 1spot: 1.-3. CARBON3, 4.-6. CARBON5, X. spot PBS instead of protein.



b) 2 µl/spot, 1spot: 1.-3. CARBON3, 4.-6. CARBON5, X. spot PBS instead of protein.

Figure 4.21 The first set of the experiment: a) after scanning b) after scanning and inverting.

a) 1 µl/spot, 2spots: 7.-9. CARBON3, 10.-12. CARBON5, X. spot PBS instead of protein.

b) 1 µl/spot, 2spots: 7.-9. CARBON3, 10.-12. CARBON5, X. spot PBS instead of protein.

Figure 4.22 The second set of the experiment: a) after scanning b) after scanning and inverting.

Table 4.5 The intensities of each spot of CARBON3 and CARBON5

Set	Sample	Intensity of protein 50 ng/ml	Intensity of protein 100 ng/ml	Intensity of protein 500 ng/ml	Intensity of protein 1000 ng/ml
1	CARBON3	75.49	78.11	67.65	73.18
1	CARBON5	87.28	80.71	82.12	70.75
2	CARBON3	89.56	85.63	90.47	92.75
2	CARBON5	103.29	120.70	99.72	107.11
1&2	X	85.72	68.18	206.26	107.22

From the result of the first set, when spotting the protein and the carbon conjugate at 2 µl/spot, 1spot, the color intensity of CARBON5 was insignificantly greater than CARBON3. Moreover, with naked eyes, the intensities were quite similar. Likewise, the result of the second set showed the same conclusion. When comparing these results to membrane X, which was spotted only PBS instead of protein, the color intensity was quite similar. No matter what concentration of protein was, the color intensity was not significantly different. In theory, the color intensity depends on the concentration of the protein; if the protein has high concentration, the color intensity always gives a high value of intensity [27]. Therefore, the color intensity, which was measured, was just the color of the carbon stain on the surface of membrane that could not be washed. The

intensity did not represent the binding between the antibody-carbon conjugate and the protein.

4.2.2.2 Label: fluorescence

4.2.2.2.1 Varying anti-NGAL antibody concentration 0.4 µg/ml and test the detection of protein on Nitrocellulose membrane by dot blot

In this study, the antibody solution was prepared using 0.4 µg/ml of anti-NGAL antibody, which was the recommended concentration for fluorescence detection. The concentrations of NGAL protein in PBS were varied from 50, 100, 500 to 1000 ng/ml and each concentration was spotted by slowly pipetting 0.5 and 1 µL/spot onto different locations of the nitrocellulose membrane and air-dried for 1 hour. The remaining non-specific site was blocked by immersing the membrane into a solution 5% (w/v) BSA in 0.1 M PBS and dry for 1 hour. After that, the membrane was spotted by slowly pipetting 0.5 and 1 µL/spot and incubated at room temperature for 1 hour. The membrane was washed with PBS and then fluorescence labeled streptavidin was spotted on the membrane 0.5 and 1 µL/spot and incubated at room temperature for 1 hour. The membrane was washed with PBS, air-dry and scan signal by florescence scanner.

In this experiment, there were two volumes of spot which were 0.5 µl/spot and 1 µl/spot. Half a microliter per spot was the amount of spot in the glass slide system and then was applied in this study. One µl/spot was the amount of spot in membrane system. The result is shown in Figure 4.23.

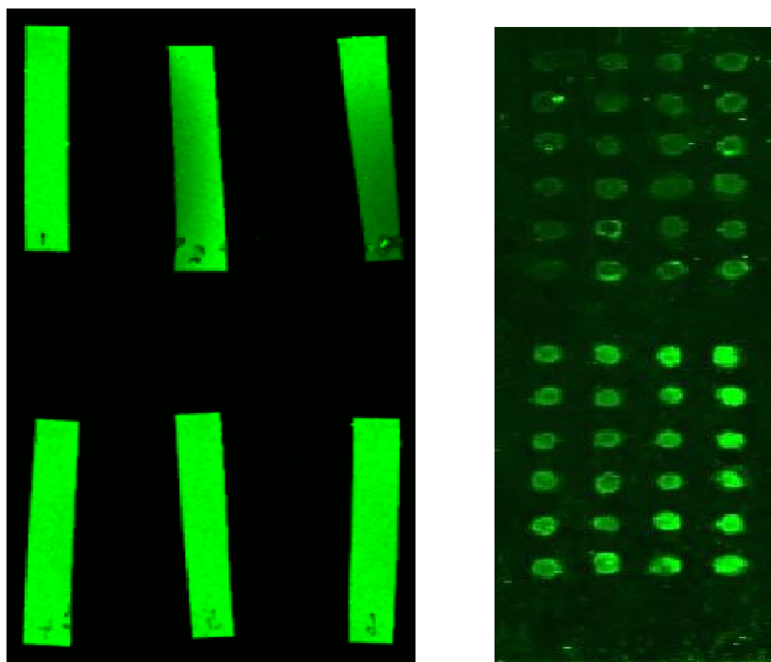


Figure 4.23 The scanning and inverting photo: membrane (left) and glass slide (right).

From this result, there was no visible spot. The membrane was a white sheet, not transparent like a glass slide. When the membrane was scanned, the spots could not be visible due to strong background signal. Therefore, the membrane system was not suitable for the fluorescence detection.