Binding Characteristics Study for Dengue Virus Non-Structural Protein 1 of Antigen and Its Antibody by Using Circular Dichroism Technique

N. A.Yusoff[1, 2], *Student Member IEEE*, N. Soin[3], *Member IEEE*, N. Abd. Rahman[4],
R. N. Z. Raja Abd. Rahman[5], M.K.B. Abd. Kahar[6], F. Ibrahim[1], *Member IEEE*
[1] Dept. of Biomedical Engineering, Faculty of Engineering, University Malaya, K.L.
[2]Biomedical Electronics Eng. Program, School of Mechatronic Eng, University Malaysia Perlis (UniMAP), Perlis.
[3] Dept of Electrical Engineering, Faculty of Engineering, University Malaya, K.L.
[4] Dept of Chemistry, Faculty of Science, University Malaya, K.L.
[5] Faculty of Biotechnology and Bimolecular Science, University Putra Malaysia, Selangor.
[6] Department of Medical Microbiology, Faculty of Medicine, University Malaya, K.L.
Email:aizas_05@yahoo.com.

***Abstract*- This paper presents the binding characteristics study of dengue non-structural protein 1 (NS1) antigen and its antibody using circular dichroism technique in far UV region. Circular dichroism (CD) is a spectroscopic technique which measures differences in the absorption of left-handed and right handed circularly polarized light. The CD spectrum can determine conformation of the NS1 antigen and its antibody, conformational changes of the antigen-antibody interaction and estimates the secondary structure of these proteins in far UV region. Firstly, CD spectrum of individual solutions of the antigen and the antibody were measured. Then, the solutions were mixed to produce a solution of complex dengue NS1 antigen and its antibody for measurement. The findings show that the antibody has the highest positive band of CD intensity follow by the complex antigen-antibody and antigen. The antibody is a chiral structure, has high helical conformation and more ordered epitope structure. Meanwhile, the NS1 antigen shows the negative and the lowest CD spectrum. The antigen is low chirality and has more random-like conformation. The complex (binding of the antigen and antibody) has the CD spectrum's shape similar to the antibody but in lower intensity. So, it has helical and beta conformations lower than the antibody. The binding characteristics of the complex solutions were also studied with increased in incubation time and with varied rotation applied. It is found that the immunoreactions between the antigen and its antibody are rapid processes which do not require too long incubation time. Besides, the applied rotation can increased the immunoreaction process.**

I. INTRODUCTION

Circular dichroism (CD) is a spectroscopic technique which measures differences in the absorption of left-handed and right handed circularly polarized light ($\Delta A = A_L - A_R$) [1]. The absence of regular structure results in zero CD intensity, while an ordered structure results in a spectrum which can contain both positive and negative signals (Fig. 1). In principle, CD can be measured for any frequency of electromagnetic radiation. While, in practice most CD involves UV or visible light. CD generally report in term of ellipticity (θ) in degrees where $\theta = 32.98\Delta A$ [2]. A CD signal will be observed when a chromophore is chiral (optically active) for the following reasons: a) intrinsically chiral because of its structure, b) covalently linked to a chiral center in the molecule, c) it is placed in an asymmetric environment due to the 3-dimensional structure

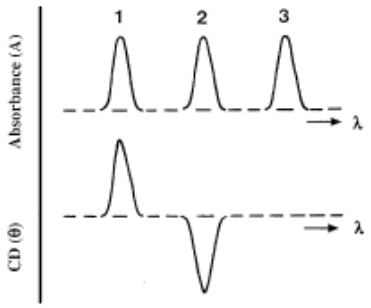

Figure 1. The relationship between absorption and CD spectra. Band 1 has a positive CD spectrum with L absorbed more than R; band 2 has a negative CD spectrum with R absorbed more than L; band 3 is due to an achiral chromophore. (Adopted from Kelly *et al*. 2005).

adopted by the molecule [2]. CD performs structural studies under the condition of protein operation (in solution) and measures the rate of structural changes of protein. Some of CD applications are estimation of protein and nucleic acid conformation, determine of conformational changes due to the interaction of asymmetric molecules (protein-protein interaction), determine of the thermodynamic of folding and unfolding of protein and determine binding constant.

Dengue virus is one of the most significant human viral pathogens transmitted by aedes mosquitoes and causes millions or more cases of infection worldwide each year, and resulting thousands of deaths. In Malaysia, 12,933 of dengue cases have been reported until 21 March 2009 with 35 deaths which is 49% higher compare to the year before within the same period of time which is 8,666 cases with 16 deaths [3]. Therefore, many studies have been done on dengue virus include to understand its structure, immunological response, vaccine, diagnosis and vector control. It is found that for dengue virus, its non-structural protein 1 (NS1) is useful for early detection of dengue which is thought to be involved in viral RNA replication [4].

In this study, the binding characteristics between antigen and antibody of dengue NS1 were investigated using circular dichroism technique in far UV region. This study differentiates CD spectra between three different solutions which are NS1 antigen (Ag), NS1 antibody (Ab), and binded NS1 antigen-antibody complex (Ag-Ab). This determines the conformation of the Ag and Ab,

conformational changes of the antigen-antibody interaction and estimates the secondary structure of the proteins in far UV region. In addition, the binding characteristics of the Ag and Ab were studied with increased in incubation time and with varied rotation applied. These results will be useful in designing a new rapid detection system for dengue NS1 enzyme-linked-immunoassay (ELISA) of lab-on-a-disk platform. In conventional ELISA method, time required for immunoreaction of the Ag and Ab takes almost 60 to 90 minutes. It uses microwell plate as a platform for reaction and required longer time for sample diffusion and mixing. Meanwhile study by Rossier *et al.* 2000 [5] stated that immunoreaction is a rapid process which does not require too long incubation time. In the lab-on-a-disk platform, the reaction will be faster due to rotation applied, reduced diffusion length and use of small volume [6]. Therefore, this study will evaluate experimentally the minimum incubation time required for immunoreactions of the antigen and antibody using CD technique. Besides, it is hypotheses that applied rotation to the mixture of the antigen and antibody will make the reaction become faster as compare to the conventional ELISA procedures which the mixture react by self diffusion.

II. Materials and Methods

Antigen, antibody and buffer preparation: The antigen used is dengue virus NS1 glycoprotein (1mg/ml, >90% purity) which consist of peptide recombinant full length protein of dengue virus. For the antibody, mouse monoclonal [DN3] to dengue virus NS1 glycoprotein was used [7]. The Ag and Ab used are from Abcam plc, Cambridge, UK. The antibody's immunogen is full length native protein purified from dengue virus 2 infected supernatant and can reacts with all types of dengue virus [8]. Since the Ab is not purified, its concentration cannot be determined in the product and is assumed similar to the antigen (1mg/ml). Both materials are in liquid form and kept in 4^0C. Both Ag and Ab liquids were centrifuged at 12000rpm for 20s in eppendorf tube prior to use to avoid the product remain in the cap. Phosphate buffer 10mM, pH7 was prepared as diluents for the Ag and the Ab. It is the most recommended buffer to be used for CD. It shows of low chirality and is not affecting CD spectra of the Ag and Ab. Several dilutions of each of the Ag and Ab were prepared in 2ml eppendorf tube for testing (example from: 100µg/ml, 50µg/ml, 20µg/ml to 4µg/ml).

Circular Dichroism instrument setting: CD machine (Jasco, model: J-810) was turned on for 30 minutes to warm up and achieve stability. A quartz cuvette size of 0.1cm (300-400µl) was used to test for samples at temperature; 20^0C. Three scans were accumulated to improve signal to noise ratio and the data is expressed in terms of ellipticities [θ] (milidegrees). The spectra were measured between 190nm and 260nm for far UV test using a spectral bandwidth of 1nm and a scan speed of 50nm/min. Spectral Manager analysis software was used to see the CD spectra and estimates the secondary structure with reference CD data from Yang (available in the software of the machine).

Circular Dichroism sample measurement: For each of the measurement, 300µl of the sample was pipetted into the cuvette and tested in the CD machine. The phosphate buffer solution was first recorded before starting with the samples to check that its absorbance is not a problem. Then, CD was tested on samples of the Ag and Ab individually for different dilutions. This is to get the best concentration of the samples for further analysis with acceptable high tension voltage (HTV). The HTV must be in the range of 400V to 600V at 190nm in order to accept CD spectra of the sample. The pattern of HTV must be traced to identify any artefacts arising from excessive absorbance [2]. After obtaining the best concentrations of the Ag and Ab, they were tested for the binding characteristics study. In this test, the Ag and Ab solutions (1:1 ratio) were mixed in 2ml eppendorf tube and 300µl of the mixture was pipetted into the cuvette for CD test. This will give CD spectra of the Ag and Ab when they were binded, and effect of binding with varied time and rotation. The estimations of secondary structure of the samples were determined in the analysis software. The complex Ag-Ab binding was also tested on equal and different ratio of each Ag and Ab used.

III. Results and Discussions

CD spectra of all of the samples were studied in far UV region which is from 190nm to 260nm [9]. They are useful for secondary structure estimations of the proteins. The phosphate buffer (10mM, pH 7) shows a very low CD spectrum which is from -0.2 to 0.5mdeg with HTV value around 380V at 190nm. The low HTV demonstrates a little absorbance of the buffer in CD spectrum and validates that the CD spectrum will not affect data of the samples. So, the phosphate buffer is very suitable to be used as diluents for the CD study.

For the samples measurements, several dilutions of the antigen and antibody have been examined to get reliable CD spectra based on its HTV value. HTV is roughly proportional to absorbance. If the HTV goes above 600V then the detector is saturated and the amplitude of the spectrum will oscillate wildly [10]. This is clearly shown in Fig. 2, for 100µg/ml of antigen, the spectrum gives more artefacts (up to 5mdeg) due to excessive absorbance with HTV around 1000V. Thus, 5µg/ml was selected for binding analysis due to the HTV is in the range from 400V to 600V. Concentration of 10µg/ml was not selected since it is outside the acceptable range and the CD spectra give some artefacts.

Meanwhile Fig. 3 shows the CD spectra and their HTV value for three different concentrations of the antibody. 20µg/ml gives a reliable CD data for binding analysis compare to the others. 50µg/ml gives excess absorbance while for 10µg/ml gives lower absorbance.

Fig. 4 shows the CD spectra of dengue NS1 antigen (5µg/ml), the antibody (20µg/ml) and the complex antigen-antibody binded. In far UV region, the CD spectrum for the Ab is between 10.50mdeg to -9.22 mdeg. The high positive intensity of CD spectrum at 190nm to 200nm shows that the Ab adopted of α-helixal conformation [11]. The broad negative band spectrum around 200nm – 240nm indicates a

mixture of β-sheet and β-turn conformers for the Ab [16]. The Ab has more ordered epitope or chiral structure that gives high CD intensity compare to the Ag and the complex Ag-Ab. Meanwhile for the antigen, the CD spectrum is between -0.47 to -2.03mdeg. The CD spectra for the Ag is more linear, very low intensity and in negative band. This shows that the Ag has very low helical structure and high of random-like coil conformation [11].

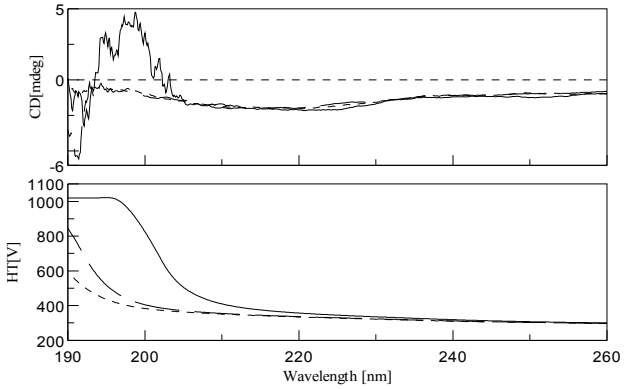

Figure 2. CD spectra and HTV value for three different concentrations of the NS1antigen: the solid line, 100µg/ml; long dashed line, 10µg/ml; the dotted line, 5µg/ml.

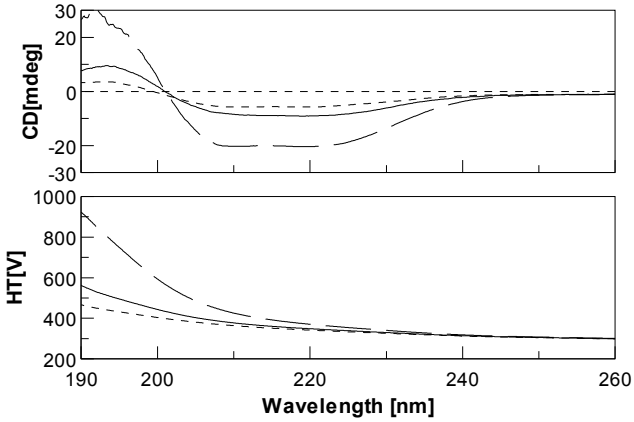

Figure 3. CD spectra and HTV value for three different concentrations of the NS1 antibody: the long dashed line, 50µg/ml; the solid line, 20µg/ml; short dashed line, 10µg/ml.

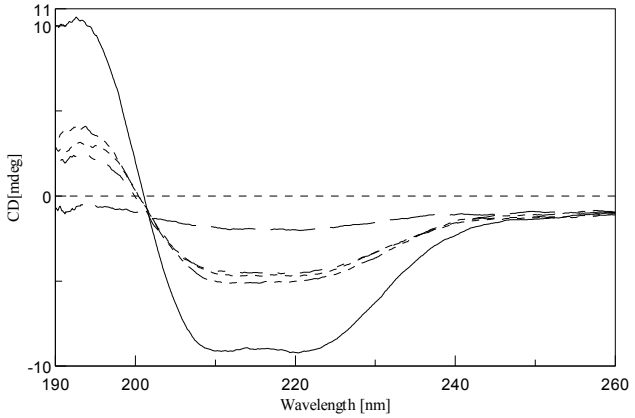

Figure 4. CD spectra for the antigen, the antibody, and the Ag-Ab binded. Antibody, the solid line; antigen, the long dashed line; antigen and antibody binded (15min), the dotted line; binded (60min), the dashed double-dotted line; binded (120min), the dashed dotted line.

When the Ag and Ab were mixed and incubated for 15 minutes, the CD spectra is between 3.16mdeg to -4.71mdeg, while for 60 minutes is between 4.10mdeg to -5.12mdeg, for 120minutes is between 2.47 to -4.57 (Fig. 4). The result shows that when the complex was formed, the CD spectrum intensity is in between the value of the single Ag and Ab with range from 4 to -5mdeg. The complex is more likely to duplicate the CD signal of Ab but in lower intensity. The large changes both in the Ag and Ab spectral region reflect a strong interaction between the Ag and Ab. The complex also adopted helical and beta structure conformation but in ratio lower than Ab. The Ab and the complex Ag-Ab is a left-handed helix as they give positive ellipticity of CD spectra at 190nm (wavelength for helixal structure). The Ag is a right-handed helix since it gives negative ellipticity [1].

In addition, at wavelength from 190nm to 240nm (Fig.4), the resultant CD intensity of the complex Ag-Ab in decreased order for different incubation time is 60min, 15min, 120min. There is not much difference shown here when they were binded with different incubation time. This is because, the chirality of the complex has a certain range of ellipticity (4 to -5mdeg). However, longer incubation might shows of complete binding for the complex (incubation at 120min). In overall, the CD spectra in the far UV region shows the absorption for 240nm and below is due to peptide bond, there is a weak but broad $n \rightarrow \pi^*$ transition centered around 220nm and more intense $\pi \rightarrow \pi^*$ transition around 190nm [2].

Kelly *et al.* 2005 said that most biological studies if not all have very small CD signals *i.e.,* ellipticities are typically in the range of 10mdeg corresponding to a difference in absorbance (ΔA) of the order of 3×10^{-4}. This is consistent with the CD spectrum of the NS1 Ab. Antibodies are heavy (~150kDa) globular plasma proteins that are also known as immunoglobulins. They have sugar chains added to some of their amino acid residues [12]. In other words, antibodies are glycoproteins. In this study, monoclonal antibody IgG (80kDa) was used which is the immunoglobulin (Ig) monomer containing only one Ig unit that specific to dengue NS1.

Meanwhile, the NS1 antigen has very low CD spectra because it is too small protein and a component of RNA in dengue virus. It is the first non-structural protein in the dengue polyprotein following envelope protein and preceding the NS2A protein. Dengue NS1 is a 46kD glycoprotein, with two glycosylated asparagines, and 12 cysteines that form 6 disulfide bridges [13]. It forms a hexamer in secreted form [14]. Mature dengue NS1 has 352 amino acid residues in the base polypeptides [13]. A CD spectrum of ribonucleosides which is a component of RNA of rice dwarf virus has been shown is in between 0 to ±1mdeg [1]. This is agreeable with the Ag that gives very low CD spectrum. Besides, organic cofactors such as flavin and haem show little of signal but if bound to their protein partner in sites, they will show chirality [2]. This condition happens to the Ag which shows very low chirality but when binds to its Ab, it gives more value of CD spectra.

The optical activity of a protein is the sum of the individual contribution from the monomer units and the

contribution from their interaction in polymer arrangement. By comparing the CD of the native polymer to that of its monomeric units, their separate contribution can be determined experimentally [1]. In this study, the contribution of each of the single Ag and Ab can be seen to form the complex. The CD spectrum of the complex is almost similar to the Ab but is reducing due to the Ag binding. Mason et al. 1990 [15] found that NS1 MAb reacted with several determinants clustered near the N terminus of the NS1 protein for amino acid from 57 to 126. More details analysis on dengue NS1 antigen cannot be done due to the absence of crystallize structure found for the antigen.

Table 1. Estimation of secondary structure's ratio (helix, beta, turn and random coil) of the Ag, Ab, and Ag-Ab from the CD spectra.

	Ab 20μg/ml	Ag 5μg/ml	Ag-Ab (15min)	Ag-Ab (60min)	Ag-Ab (120min)
Helix	35.9%	24.2%	33.1%	33.2%	31.5%
Beta	7.3%	0.0%	0.5%	2.6%	0.0%
Turn	22.8%	32.9%	29.3%	27.5%	29.9%
Random	34.0%	42.8%	37.2%	36.7%	38.6%
Total	100.0%	100.0%	100.0%	100%	100.0%

Several forms of secondary structure are present in native proteins. They include a repetitive structures such as helix, parallel and antiparallel β-pleated sheets, and β-turn [1] which are approximately, one half of an average globular protein. The remainder of polypeptide chain is described as having a loop or random coil conformation. The estimation of secondary structure was done by comparing the CD data with reference data from Yang (available in the CD spectrum analyzer software). From Table 1, when the Ag and Ab were binded, the helical ratio of the complex was lower than the single Ab and higher than the single Ag. Antibody has the highest helical ratio that gives high CD spectrum compare to others. This result further confirms the CD conformation analysis that has been described from the CD spectra. For Beta ratio, the complex almost follows the ratio of the antigen which is almost 0%. For β-turn, the complex has higher β-turn than the Ab but lower than the Ag. The Ag alone has high random-like coil conformation followed by the complex, and lastly the Ab. The ratio of the secondary structure estimations of the complexes have shown not much difference with increased in incubation time.

To observe experimentally the minimum incubation time required for immunoreactions, the Ag (5μg/ml) and Ab (20μg/ml) were mixed in 1:1 ratio with 750rpm applied rotation and read in CD machine from 1, 5, 10, and 15minutes. Fig. 5 shows the effect of binding for the Ag and Ab incubated from 1 to 15min. The CD spectra intensity are slightly differ at wavelength from 190nm to 200nm (helixal structure ratio), which in decreased order are 5min, 10min, 15min, 1min. From the result with increased in incubation time, the helical conformation will decrease and give less CD intensity. But for 1min incubation, the lowest

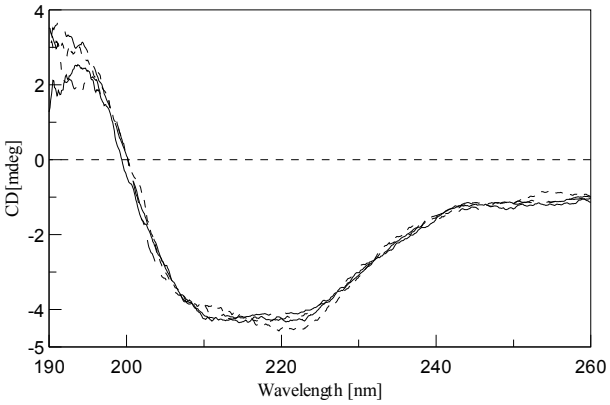

Figure 5. CD spectra for antigen and antibody binding with different time of incubation: the solid line, 1min; the long dashed line, 5min; the dotted line, 10min; the dashed dotted line, 15min.

CD intensity can be assumed due to the availablity of Ag in the solution. However, the CD spectra have shown no significance difference at wavelength from 200 to 260nm (Beta and random-like coil conformation) which revealed that there is not much conformational changes occur in that region with an increased of time.

The CD intensity of the complex with incubation time varied from 1-15min (Fig.5) are in range between 4 to -4.5mdeg. It is similar to the CD intensity of the complex with incubation from 15-120min which is range between 4 to -5mdeg. Therefore, it suggests that the Ag and Ab solutions were binded immediately after they were mixed. This happened because both the Ag and Ab solutions mixed and binded easily with the applied rotation. From the result, it shows that immunoreaction is a rapid process and does not require too long incubation time as there is no significance difference for CD spectra of the Ag-Ab that incubated from 1 to 15min. This is supported by Rossier *et al.* 2000 which stated that immunoreaction is a rapid process.

In order to study the effect of applied rotation to the reaction of the Ag and Ab, the solutions of 5μg/ml Ag and 20μg/ml of Ab were mixed in 1:1 ratio and separated for three different conditions (no rotation, rotate at 370rpm, and rotate at 750rpm). They were incubated for 15 minutes prior to test. Fig. 6 shows that for rotation at 750rpm, the CD spectrum has the lowest intensity and lowest helical conformation. It might shows of complete binding as having the lowest helical conformation as shown by the CD of the complex that incubated for 120min. For rotation at 370rpm and no rotation applied (mixing through pipetting), the CD spectra have shown no significance difference. However at 190nm to 200nm (helical structure), 370rpm shows lower value of CD (more binding) than without rotation applied. The rpm is selected according to Lai *et al.* 2004 that mix Ag and Ab at 375rpm and 760rpm for application of ELISA on the a lab-on-a-disk. Generally, rotation will increase the reaction rate as the solutions are mix completely. If the platform for reaction has high diffusion length, it will take long time for reaction of non-mix solutions**.** From the result, it can be said that with the applied rotation, we can increase the immunoreaction between the Ag and Ab.

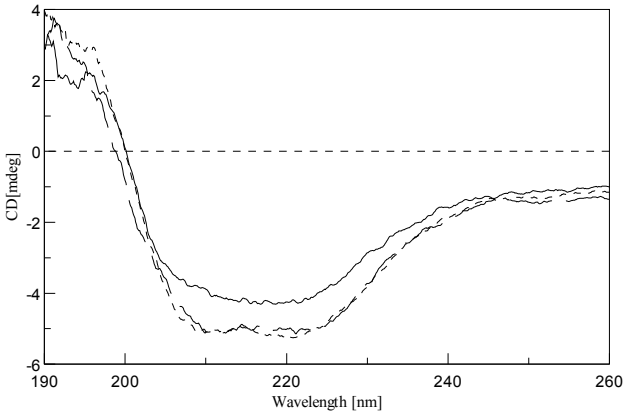

Figure 6. CD spectra for antigen and antibody binding with different applied rotation: the solid line, 750rpm; the long dashed line, 370rpm; dotted line, no rotation (mixing through pipetting).

Since the Ab is not purified, its concentration cannot be determined. So an analysis is done to see the effect of different volume ratio used for the Ag and Ab reaction. From Fig. 7, the CD spectra show that when more volume of Ab is used, the complex has the highest CD intensity. While if more Ag is used, the complex has the lowest CD intensity. For the same volume of Ag and Ab used, the CD intensity is in the middle of the two signals. Therefore from the result, the different volume ratio used for the Ag and Ab binding will cause the CD spectra intensity decreased or increased. However, the CD intensity is still in the range of the complex Ag-Ab binding which is between the ranges of 4 to -5mdeg.

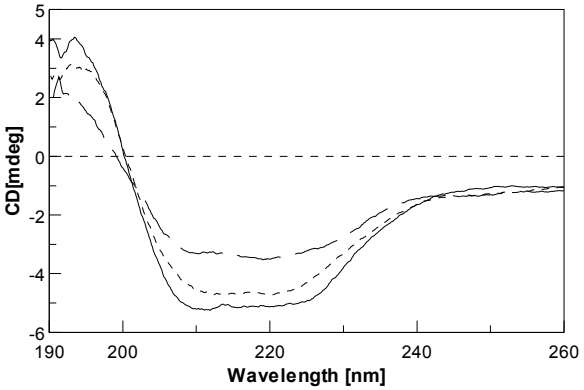

Figure 7. CD spectra for antigen and antibody binding with different ratio of Ag:Ab : the solid line, 1:2 ratio; the long dashed line, 2:1 ratio; dotted line, 1:1 ratio.

IV. Conclusions

Circular dichroism spectra in far UV region can show the conformational changes of the antigen and antibody when they were binded. Dengue NS1 antibody gives the highest CD intensity compares to the antigen and the complex. So, it has the highest chirality structure and high helical conformation. The NS1 antigen has the lowest chirality structure, high random coil conformation and will improve its CD spectrum when it is binded to the antibody to form the complex Ag-Ab. The complex has the CD spectra intensity in range from 4 to -5mdeg and adopted the helical and beta conformation in ratio lower than the antibody. The results of varied time and varied rotation applied indicate that immunoreaction is a rapid process which does not require too long incubation time and the applied rotation can increased the immunoreaction process. Thus, these results can lead to a new design of a rapid detection system for dengue NS1-ELISA on lab-on-a-disk platform with incubation time of less than 15min and applied rotation up to 750rpm.

Acknowledgments

N.A. Yusoff would like to thank University Malaysia Perlis and Ministry of Higher Education, Malaysia for supporting her study. The research is funded by University of Malaya's research grant (PJP-FS229-2008A) and postgraduate research fund (PPP-PS059-2008A). We acknowledge these contributions.

References

[1] I.Tinoco Jr, K. Sauer, J.C. Wang, and JD. Puglisi. *Physical Chemistry. Principle and Applications in Biological Sciences*, 4th ed.., Prentice Hall, 2002, pp. 573-575.

[2] S.M. Kelly, T.J. Jess, and N.C. Price, "How to study proteins by circular dichroism," *Biochimia et Biophysica Acta 1751*, pp. 119-139, July 2005.

[3] IT Unit Taiping Hospital. "Ministry of Health estimates less than 1000 dengue cases per year". [Online]. 2009 April 14 [cited 2009 Aug 28]. Available from: http://htaiping.moh.gov.my/modules/news/article.php?storyid=18.

[4] J.M. Mackenzie, M.K. Jones, and P.R. Young, "Immunolocalization of the Dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication," *Virology*, vol. 220, pp.232-240, 1996.

[5] J. S. Rossier, G. Gokulrangan, H. H Girault., S. Svojanovsky, and G.S. Wilson, "Characterization of Protein Adsorption and Immunosorption Kinetics in Photoablated Polymer Microchannels," *Langmuir: the ACS journal of surfaces and colloids,* vol.16, no.22, pp. 8489-8494, 2000.

[6] S. Lai, S. Wang, J. Luo, J. Lee, S.T. Yang, and M.J. Madou, "Design of a Compact Disk-like Microfluidic Platform for Enzyme-Linked Immunosorbent Assay," *Annal. Chemistry,* vol. 76, pp. 1832-1837, April 2004.

[7] Abcam plc. Product Documentation. "Dengue Virus NS1 glycoprotein protein (ab64456)". [Online]. [cited 2009 Aug 28]. Available from: http://www.abcam.com/Dengue-Virus-NS1-glycoprotein-protein-ab64456.html.

[8] Abcam plc. Product Documentation. "Dengue Virus NS1 glycoprotein antibody [DN3] (ab41616)". [Online]. [cited 2009 Aug 28]. Available from:http://www.abcam.com/Dengue-Virus-NS1-glycoprotein-antibody-DN3-ab41616.html.

[9] N. Sreerama, S. Yu, Venyaminov, and R.W. Woody, "Estimation of Protein Secondary Structure from Circular Dichroism Spectra: Inclusion of Denatured Proteins with Native Proteins in the Analysis," *Analytical Biochemistry*, vol.287, pp. 243-251, April 2000.

[10] J. Douglas, "An Introduction to Circular Dichroism". [Online]. [cited 2009 Aug 25]. Available from: http://mass-spec.chem.cmu.edu/procedure_files/cd/cd_guide.doc.

[11] I. Laczko, S. Bottka, G.K. Toth, C. Malvy, J.-R. Bertrand, and M. Hollosi, "Interaction of fusogenic peptides with an antisense oligonucleotide in solution and in the presence of micelles: conformational studies," *Biochemical and Biophysical Research Communications*, vol. 313, no. 2004, pp. 356-361, November 2003.

[12] T. Mattu, R. Pleass, A. Willis, M. Kilian, M. Wormald, A. Lellouch, P. Rudd, J. Woof, R. Dwek, "The glycosylation and structure of human serum IgA1, Fab, and Fc regions and the role of N-glycosylation on Fc alpha receptor interactions". *J Biol Chem* vol. 273 no. 4, pp. 2260–2272, 1998.

[13] T.P. Wallis, C.Y. Huang, S.B. Nimkar, P.R. Young, and J.J. Gorman, "Determination of the disulfide bond arrangement of dengue virus NS1 protein," *J. Biol. Chem*. vol. 279, pp. 20729-20741, 2004.

[14] M. Flamand, F. Megret, M. Mathieu, J. Lepault, F. A. Rey, and V.Deubel, "Dengue Virus Type 1 Nonstructural Glycoprotein NS1 Is Secreted from Mammalian Cells as a Soluble Hexamer in a Glycosylation-Dependent Fashion". *J. of. Virology*, vol. 73, no. 7, pp. 6104-6110, July 1999.

[15] P.W. Mason, M.U. Zügel, A.R. Semproni, M.J. Fournier and T.L. Mason, "The antigenic structure of dengue type 1 virus envelope and NS1 proteins expressed in Escherichia coli," *J. of General Virology*. vol. 71, pp. 2107-2114, 1990.

[16] M. J. Gómara, G. Ercilla, M. A. Alsina and I. Haro, "Assessment of synthetic peptides for hepatitis A diagnosis using biosensor technology" J. Immunological Methods, vol. 246, pp. 13-24, July 2000.