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Raman Molecular Fingerprint of Non-Structural Protein 1 in Phosphate Buffer Saline with Gold Substrate

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Abstract*— SERS is a form of Raman spectroscopy that is enhanced with nano-sensing chip as substrate. It can yield distinct biochemical fingerprint for molecule of solids, liquids and gases. Vice versa, it can be used to identify unknown molecule. It has further advantage of being non-invasive, non-contact and cheap, as compared to other existing laboratory based techniques. NS1 has been clinically accepted as an alternative biomarker to IgM in diagnosing viral diseases carried by virus of *flaviviridae*. Its presence in the blood serum at febrile stage of the *flavivirus* infection has been proven. Being an antigen, it allows early detection that can help to reduce the mortality rate. This paper proposes SERS as a technique for detection of NS1 from its scattering spectrum. Contribution from our work so far has never been reported. From our experiments, it is found that NS1 protein is Raman active. Its spectrum exhibits five prominent peaks at Raman shift of 548, 1012, 1180, 1540 and 1650cm^{-1}. Of these, peak at 1012cm^{-1} scales the highest intensity. It is singled out as the peak to fingerprint the NS1 protein. This is because its presence is verified by the ring breathing vibration of the benzene ring structure side chain molecule. The characteristic peak is found to vary in proportion to concentration. It is found that for a 99% change in concentration, a 96.7% change in intensity is incurred. This yields a high sensitivity of about one a.u. per ppm. Further investigation from the characterization graph shows a correlation coefficient of 0.9978 and a standard error estimation of 0.02782, which strongly suggests a linear relationship between the concentration and characteristic peak intensity of NS1. Our finding produces favorable evidence to the use of SERS technique for detection of NS1 protein for early detection of *flavivirus* infected diseases with gold substrate.

I. Introduction

Antibody IgM has been the most popular biomarker amongst clinicians for diagnosis of terminal diseases caused by *flavivirus* infection; non-structural protein 1 (NS1) has been listed as one of the alternative biomarkers [1]. During febrile stage of the infection, NS1 can be found in the blood serum of patients. Its presence in the serum is low, measuring 0.01 to 50ppm. Current methods to detect for the two are such as ELISA, RT-PCR [2-3], which has limitations such as being invasive, liable to blood borne infection, delayed diagnosis, tedious time consuming procedures, hi-tech high cost equipment and well-trained physicians.

Raman Spectroscopy has been discovered to be capable of revealing the molecular structure of solids, liquids and gases from its scattering spectrum since 1928. However, further development of it has been impeded by the strength of the signal. With benefits derived from the integration of nano technology and laser technology, Surface Enhanced Raman Spectroscopy (SERS) is found capable of enhancing the weak Raman signal 10^{+3} to 10^{+7} times, enabling detection up to a single molecule [4], with the use of an appropriate substrate. This immediately promotes Raman spectroscopy technique to a wide spectrum of applications, in particular, for detection of analyte in low concentration, in disease detection.

Our work here intends to establish a fingerprint spectrum for NS1, gold substrate is selected. Raman spectrum of NS1 has not been found in commercial standard spectral library. Peaks in the spectrum and molecular structure of NS1 will be investigated. In addition, the relationship between concentration of NS1 and spectral intensity will be examined.

A. Raman and SERS

Raman Spectroscopy is a technique to study the molecular structure and property of solids, liquids and gases from its scattering spectrum. The resulting spectrum offers a detailed biochemical fingerprint, which is useful to identify unknown molecule.

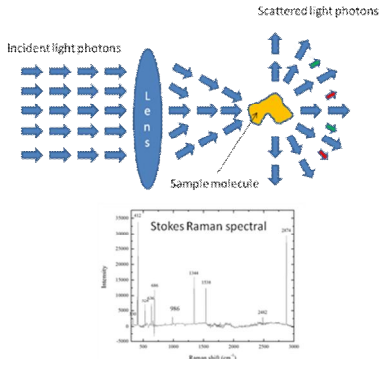

Figure 1: Big blue arrows on the scattered light photons are Rayleigh scattering, small red arrow represent Raman (stokes) photons and small green arrow represent Raman (anti-stokes) photons. Raman spectral is obtained by frequency shift of the incident laser light on the left and scattered laser light on the right

As laser is exposed onto the molecules, the monochromatic light scatters, in accordance to one of the common light phenomenon, i.e. light scattering. This forms the foundation of Raman spectroscopy. The scattering of light consists of elastic and inelastic scatter as illustrated in

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Figure 1. Elastic scatter (or aka Rayleigh scatter) is the light scattered at the same frequency of the incident laser beam, while the inelastic scatter (or aka Raman scatter) exhibits radiation at a different frequency. The difference in frequency is due to the absorption and release of energy during molecular vibration [5].

Raman spectrum displays the variation in the intensity of inelastic scattering as a function of difference in energy between the incident and the scattered photons. The difference between the initial and final vibration energy levels determines if the scattered photon is losing energy (stokes) or gaining energy (anti-stokes), analogous to the principle in potential difference. At room temperature, stokes scattering is normally stronger and measured for spectrum display. The resultant spectrum is characterized by a shift in wavelength (cm^{-1}) known as Raman shift. Every molecule has a unique Raman spectrum, which can be adopted as its very own molecular fingerprint.

Since the amount of inelastic scattering is infinitesimally small relative to that of the elastic scattering, i.e. a ratio of 1 in 10^6 to 1 in 10^{10}, hence the Raman signal is found to be extremely weak [6], which staunches its application.

In SERS, the Raman signal is amplified to a useable range, by binding molecules to the surface of noble metals, popularly silver, gold and copper. The amplification is a result of enhanced electromagnetic field and chemical enhancement produced by laser excitation of localized surface plasmon on a rough metal surface, as molecules are bounded onto metal nanoparticles (MNP), influencing the incident and scattered lights. This has empowered the Raman spectroscopy to be competent even to conduct spectroscopy on a single molecule [7, 8]. Furthermore, as strong intensity data are within its means, SERS has been demonstrated to be capable of detecting biological molecules such as proteins [9], DNA[10], RNA [11], even the entire pathogen [12].

The intensity of Raman signal from SERS depends on the size, shape, inter-particle spacing of the molecules and dielectric environment, which are highly dependent on the morphology and activity of the enhanced surface, also known as substrate [13, 14]. Types of substrates for SERS currently available are such as metal roughened electrode, metal island films, colloids and nanostructures, including nanowire, nanopillar, nanorod.

Since the advent of SERS, Raman spectroscopy has now become a widely accepted analysis technique to a variety of application. This owes to the ability of SERS to provide qualitative as well as quantitative information on the molecule. For use in the detection of diseases, Raman peaks are used to disclose the molecular structure while distinctive peak is used to earmark the biomarker of the disease. Existing works have shown successes of SERS in producing Raman spectra of infected samples from, such as cell, blood, urine, plasma, saliva and tears, to detect anomalies [15-17].

B. NON-STRUCTURAL PROTEIN 1

Dengue fever, Japanese encephalitis, Murray Valley encephalitis, Tick-borne encephalitis, West Nile encephalitis and Yellow fever are viral diseases brought by virus of *Flaviviridae (genus Flavivirus),* with fatal consequences. *Flavivirus* genome is a single strand of ribonucleic acid (RNA) which consists of nonstructural proteins NS1, amongst others [18].

NS1 protein has been listed as an alternative marker in detecting viral disease [1]. It is believed to involve in virus replication process [19]. NS1 is detectable in patient's blood serum from day 1 to 9 following the onset of disease where the amount is estimated to be in the range of 0.01 to 50ppm [2].

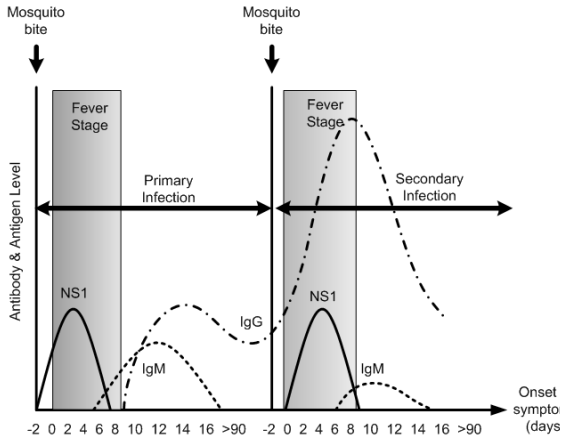

Figure 2: Kinetic of NS1, IgM and IgG in primary and secondary *flavivirus* infection

Figure 2 shows the kinetic of NS1, IgM and IgG in primary and secondary infection of dengue fever, as an example of *flavivirus* infection. It can be observed that the presence of NS1 comes before IgM and IgG. This explains its potential for early disease detection. Currently commercially available detection methods for NS1 are such as PanBio Early ELISA, Platelia NS1 assay [20-24].

II. METHODOLOGY

A. Sample Preparation

Flavivirus NS1 glycoprotein protein (Ab64456) in liquid form at a concentration of 100mg/ml (1000ppm) from Abcam is used as received. For diluting the protein into 500ppm, 200ppm, 50ppm, 20ppm and 10ppm, phosphate buffer saline (PBS) of pH7 is used as solvent. Firstly, the uniformly stirred and diluted protein is incubated at 4°C for 24 hours. Then 5µL sample of each concentration is directly deposited onto the gold substrate and left dried before Raman analysis.

B. Equipment

The spectra acquired from this study are obtained from Perkin-Elmer Raman Station 400F dispersive Raman spectrometer. It excitation source is near infrared 785nm laser. The spectrometer was set to 10% of full power, approximately 10mW on the sample. The detector used is a highly sensitive open electrode CCD detector, which is subjected to a small random current known as cosmic ray. This current appears as random sharp features superimposed on the Raman spectra [25]. To reduce its occurrence, cosmic ray removal mode with median filter is enabled. To reduce

fluorescent effect, it is necessary to enable the baseline correction mode. Spectra shown are averaged spectra from 3x3 point maps, with each point collected at an acquisition time of 5 seconds, unless specified otherwise.

III. Results and Discussion

A. Molecular Fingerprint of NS1 Protein at 1000ppm

Results of Raman analysis from repeated testing on gold substrate establish that NS1 protein is sufficiently Raman active to produce a molecular fingerprint.

Sample of 5uL NS1 protein at a concentration of 100mg/ml (1000ppm) is deposited onto the gold coated slide.

Figure 3 displays the averaged Raman spectrum from 3x3 points map of NS1 protein. Five prominent peaks representing NS1 protein can be observed at Raman shift of 548, 1012, 1180, 1540 and 1650cm^{-1}. Further investigation reveals their relationship to vibration bands of molecular structure of NS1 protein, which reflects the secondary structure of the protein.

Of these, peak 1012cm^{-1} has the highest intensity. It is selected to be the characteristic peak of NS1 protein. This is because the peak is also observable in L-phenylalanine (Phe) and L-tryptophan (Trp) acid amino, confirming the presence of benzene-ring in the structure of NS1 side chain molecule. The peak is attributed to ring breathing vibration of the trigonal ring of benzene ring structure[26].

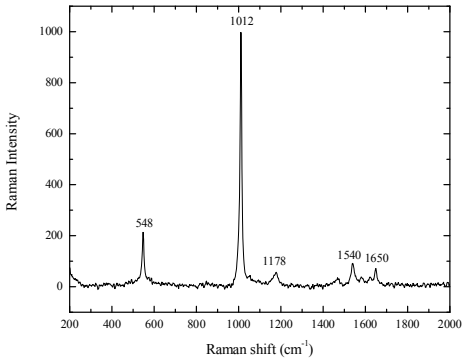

Figure 3: Raman spectra of NS1 protein at 1000ppm deposited on gold substrate

B. Characteristic Spectral Peak Intensity of NS1 with Concentration

The second part of our experiment investigates the characteristic peak intensity as NS1 concentration is reduced. NS1 protein at a concentration of 100mg/ml (1000ppm) is prepared into 6 different grades of concentration: 1000ppm, 500ppm, 200ppm, 100ppm, 50ppm and 10ppm. The Raman spectra of these samples are then measured and analyzed on gold coated slides.

Spectra of NS1 at different grades of concentration as shown in Figure 4 suggests positively that characteristic peak at 1012cm^{-1} provides a distinctively good fingerprint for NS1, since it is clearly visible, even up to 10ppm.

With reference to Figure 4, intensity of the characteristic peak is observed to increase with concentration. With just a blank gold coated slide, the Raman spectrum displays only the background, with no visible activity. As concentration is increased from 10ppm to 1000ppm, the intensity of characteristic peak also increases from 33a.u to 1000a.u. In other words, for a 99% change in concentration, a 96.7% change in intensity is incurred. This yields a sensitivity of about one a.u. per ppm. The increase is due to the amount NS1 molecules present at the higher level of concentration are more, inducing higher molecular vibration and thus more scattering. Similar finding is found in previous works on melamine [27- 28].

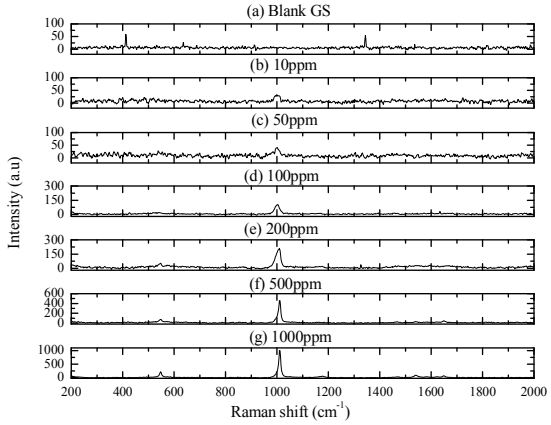

Figure 4: Raman spectrum of NS1 on gold substrate at different concentration

In addition, a slight move to the left in Raman shift is also observed in the characteristic peak as concentration is reduced. At concentration of 1000ppm, the maximum peak is recorded at 1012cm^{-1}; whereas at concentration of 10ppm, the peak has moved to 1000cm^{-1}. This indicates a shift of 12cm^{-1} for 990 change in ppm, or 0.01212cm^{-1} per ppm, which is tolerable and observed to exhibit in other works as well [29].

C. Characterization of NS1 Raman Spectra with Concentration

The previous section illustrates the relationship between characteristic peak intensity and concentration of NS1. Here, the relationship is explored further using linear regression analysis.

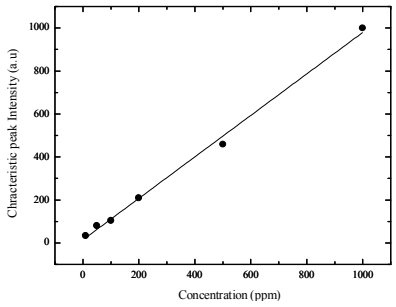

Figure 5: Characterisation of NS1 versus concentration

From the graph as shown in Figure 5, the correlation coefficient (R) is found to be +0.9978. Correlation coefficient value close to +1 value indicates a strong positive fit. This strongly suggests a linear relationship between the

concentration and characteristic peak intensity of NS1. The standard error estimation at the estimated slope is 0.02782, showing only a small variation in the slope of regression line from different concentrations. This finding accentuates further evidence from Section IIIB to use of SERS technique for detection of NS1 protein, with gold substrate.

IV. Conclusion

This paper has presented results from a maiden attempt in experimenting detection of NS1 protein with SERS technique, gold being the substrate. Our results demonstrate that NS1 protein is Raman active, with 5 prominent peaks at Raman shift of 548, 1012, 1180, 1540 and 1650cm^{-1}. These peaks are found related to the secondary structure of the NS1 protein. Of these, peak 1012cm^{-1} is selected as the molecular fingerprint for NS1 protein, as it is confirmed to have been contributed by the ring breathing vibration of the benzene ring structure side chain molecule and its intensity is the highest of all. It is also found that the characteristic peak intensity increases with concentration of NS1 sample. The peak is noticeable even up to a concentration of 10ppm. Based on the correlation coefficient and standard error estimation from linear regression analysis of the characterisation graph, the characteristic peak intensity is found to vary linearly to concentration. This finding is especially desirable to use of SERS technique for detection of NS1 protein for early detection of *flavivirus* infected diseases.

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References

[1] WHO, "Dengue: guidelines for diagnosis, treatment, prevention and control -- New edition.," in Chapter 4: Laboratory diagnosis and diagnostic tests, ed. France: WHO Press, 2009.

[2] S. Alcon, et al., "Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections," Journal of Clinical Microbiology, vol. 40, pp. 376-381, 2002.

[3] S. Datta and C. Wattal, "Dengue NS1 antigen detection: A useful tool in early diagnosis of dengue virus infection," Indian Journal of Medical Microbiology, vol. 28, pp. 107-110, 2010.

[4] S. L. Kleinman, et al., "Creating, characterizing, and controlling chemistry with SERS hot spots," Physical Chemistry Chemical Physics, vol. 15, pp. 21-36, 2013.

[5] C. V. Raman, "A change of wave-length in light scattering [8]," Nature, vol. 121, p. 619, 1928.

[6] M. Fan, et al., "A review on the fabrication of substrates for surface enhanced Raman spectroscopy and their applications in analytical chemistry," Analytica Chimica Acta, vol. 693, pp. 7-25, 2011.

[7] K. Kneipp, et al., "Single Molecule Detection Using Surface-Enhanced Raman Scattering (SERS)," Physical Review Letters, vol. 78, pp. 1667-1670, 1997.

[8] S. Nie and S. R. Emory, "Probing Single Molecules and Single Nanoparticles by Surface-Enhanced Raman Scattering," Science, vol. 275, pp. 1102-1106, February 21, 1997 1997.

[9] Z. A. Combs, et al., "Label-free raman mapping of surface distribution of protein A and IgG biomolecules," Langmuir, vol. 27, pp. 3198-3205, 2011.

[10] L. R. Allain and T. Vo-Dinh, "Surface-enhanced Raman scattering detection of the breast cancer susceptibility gene BRCA1 using a silver-coated microarray platform," Analytica Chimica Acta, vol. 469, pp. 149-154, 2002.

[11] J. D. Driskell, et al., "Rapid microRNA (miRNA) detection and classification via surface-enhanced Raman spectroscopy (SERS)," Biosensors and Bioelectronics, vol. 24, pp. 917-922, 2008.

[12] R. M. Jarvis and R. Goodacre, "Discrimination of Bacteria Using Surface-Enhanced Raman Spectroscopy," Analytical Chemistry, vol. 76, pp. 40-47, 2003.

[13] L. A. Dick, et al., "Metal Film over Nanosphere (MFON) Electrodes for Surface-Enhanced Raman Spectroscopy (SERS): Improvements in Surface Nanostructure Stability and Suppression of Irreversible Loss," The Journal of Physical Chemistry B, vol. 106, pp. 853-860, 2001.

[14] X. Du, et al., "Qualitative and quantitative determination of melamine by surface-enhanced raman spectroscopy using silver nanorod array substrates," Applied Spectroscopy, vol. 64, pp. 781-785, 2010.

[15] C. Anyu, et al., "Detecting narcotic usage using surface-enhanced raman spectroscopy on saliva samples," 2009, pp. 71-74.

[16] J. M. Benevides, et al., "Raman Spectroscopy of Proteins," in Current Protocols in Protein Science, ed: John Wiley & Sons, Inc., 2001.

[17] T. R. Ravindran and A. K. Arora, "Study of Raman spectrum of uranium using a surface-enhanced Raman scattering technique," Journal of Raman Spectroscopy, vol. 42, pp. 885-887, 2011.

[18] B. D. Lindenbach and C. M. Rice, "Molecular biology of flaviviruses," in Advances in Virus Research. vol. Volume 59, ed: Academic Press, 2003, pp. 23-61.

[19] M. G. Jacobs, et al., "Dengue virus nonstructural protein 1 is expressed in a glycosyl-phosphatidylinositol-linked form that is capable of signal transduction," FASEB Journal, vol. 14, pp. 1603-1610, 2000.

[20] M. D. R. Q. Lima, et al., "Comparison of two generations of the panbio dengue NS1 capture enzyme-linked immunosorbent assay," Clinical and Vaccine Immunology, vol. 18, pp. 1031-1033, 2011.

[21] A. H. Ramirez, et al., "Evaluation of dengue NS1 antigen detection tests with acute sera from patients infected with dengue virus in Venezuela," Diagnostic Microbiology and Infectious Disease, vol. 65, pp. 247-253, 2009.

[22] C. H. Tan, et al., "Evaluation of the Dengue NS1 Ag strip® for detection of Dengue virus antigen in Aedes aegypti (Diptera: Culicidae)," Vector-Borne and Zoonotic Diseases, vol. 11, pp. 789-792, 2011.

[23] P. Tontulawat, et al., "Evaluation of rapid immunochromatographic NS1 test,anti-dengue IgM tést,semi-nested PCR and IgM ELISA for detection of Dengue virus," Southeast Asian Journal of Tropical Medicine and Public Health, vol. 42, pp. 570-578, 2011.

[24] S. Zainah, et al., "Performance of a commercial rapid dengue NS1 antigen immunochromatography test with reference to dengue NS1 antigen-capture ELISA," Journal of Virological Methods, vol. 155, pp. 157-160, 2009.

[25] S. Li and L. Dai, "An improved algorithm to remove cosmic spikes in Raman spectra for online monitoring," Applied Spectroscopy, vol. 65, pp. 1300-1306, 2011.

[26] J. De Gelder, et al., "Reference database of Raman spectra of biological molecules," Journal of Raman Spectroscopy, vol. 38, pp. 1133-1147, 2007.

[27] Y. Cheng, et al., "Screening melamine adulterant in milk powder with laser Raman spectrometry," Journal of Food Composition and Analysis, vol. 23, pp. 199-202, 2010.

[28] F. Siak, et al., "Raman Spectra of Drop Coating Deposition Raman for Melamine Solution," presented at the 2012 IEEE-EMBS 2nd Conference on Biomedical Engineering (IECBES 2012), Langkawi, Malaysia, 2012.

[29] X. Li, et al., "Surface-enhanced Raman spectroscopy differences of saliva between lung cancer patients and normal people," Munich, Germany, 2011, pp. 808722-5.