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Nonstructural Protein 1 Characteristic Peak from NS1-Saliva Mixture with Surface-Enhanced Raman Spectroscopy

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Abstract— **Surface Enhanced Raman spectroscopy (SERS) is an enhanced technique of Raman spectroscopy, which amplifies the intensity of Raman scattering to a practical range with adsorption of analyte onto nano-size plasmonic material such as gold, silver or copper. This feature of SERS has given it a niche in tracing molecular structure, especially useful for marking diseases specific biomarker. NS1 protein has been clinically accepted as an alternative biomarker for diseases caused by *flavivirus*. Detection of Nonstructural Protein 1 (NS1) will allow early diagnosis of the diseases. Its presence in the blood serum has been reported as early as first day of infection. With gold substrate, our work here intends to explore if SERS is suitable to detect NS1 from saliva, with saliva becoming the most favored alternative to blood as diagnostic fluid due to its advantages in sample collection. Our experimental results find both gold coated slide (GS) and saliva being Raman inactive, but the molecular fingerprint of NS1 protein at Raman shift 1012cm^{-1}, which has never been reported before. The distinct peak is discovered to be attributed by breathing vibration of the benzene ring structure of NS1 side chain molecule. The characteristic peak is also found to vary in direct proportion to concentration of the NS1-saliva mixture, with a correlation coefficient of +0.96118 and a standard error estimation of 0.11382.**

I. INTRODUCTION

Early diagnosis could offer a second chance of life for patients suffering from terminal diseases, by allowing early intervention and treatment. Japanese encephalitis, Murray Valley encephalitis, tick-borne encephalitis, West Nile encephalitis, dengue fever, and yellow fever are viral diseases that could lead to fatal consequences. Virus of Flaviviridae (genus Flavivirus) is found to be the cause. [1].

Recent development sees NS1 protein being used as an alternative biomarker for detecting *flavivirus* diseases, especially during the febrile stage, with methods such as ELISA and RT-PCR using blood serum as sample [2, 3]. However, these methods are found to be invasive, carry risk of blood-related infection, time consuming and tedious which results in delayed diagnosis, rely on expensive equipment and experienced physicians.

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Saliva is gaining interest gradually as a diagnostic fluid. Saliva is initially used to diagnose oral related diseases such as oral cancer, periodontitis and Sjogren's syndrome. Of late, its usage has expanded to systemic diseases, with infectious diseases included. Biomarkers for infectious diseases such as dengue[4], pigeon breeder's disease (PBD) [5] and and lyme disease [6], taken from antibodies in saliva samples have been identified and ELISA is the commonly reported method used.

Raman Spectroscopy is discovered as a mean to study the molecular structural property of solid, liquid and gases from its scattering spectrum since 1928[7]. It offers a detailed biochemical fingerprint, useful for identification of unknown molecule[7]. However, its practical application is held back owing to weakness of the signal. It is not until SERS that the technique becomes popular. SERS is a Raman analysis technique which is capable to amplify the Raman signal of the molecules by binding them with noble metal known as substrate. However the intensity of the amplified signal is highly dependent on the substrate used. Two independent publications have reported success of SERS in detection of a single molecule [8, 9] in 1997. Of works on saliva samples are such as lung cancer [10, 11], oral cancer [12], and HIV [13]. The advantages of SERS technique from biomedical perspective are: amount of sample required is minimal; preparation for spectroscopy is minimal; analysis is simple and fast; test is non-destructive and easily reproducible.

Our work here explores if SERS is suitable as a detection technique for NS1 in saliva. Since Raman spectrum of NS1 is not found yet in the commercial standard spectra library, its molecular fingerprint is first established. Then, distinct peaks in the spectrum are related to molecular structure of NS1. Next, NS1-saliva mixture at different concentration is analyzed using SERS method and the relationship between concentration and spectral peak intensity is studied.

II. SURFACE ENHANCED RAMAN SPECTROSCOPY

Fundamental to Raman spectroscopy is the scattering of light. As laser light impacts onto molecules, elastic scatter (same frequency as the incident laser beam) and inelastic scatter (different frequency as the incident laser beam) are produced. As molecule vibrates, the scattered photon could be losing energy (stokes) or gaining energy (anti-stokes). Raman spectrum displays this difference in energy between the incident and the scattered photons with variation in the intensity of inelastic scattering.

As molecules vibrate, the electric dipole moment, P which is directly proportional to it polarizability, is induced.

$$P = \alpha E \quad (1)$$

where E is the electrical field strength of the electromagnetic wave (incident light) and α is a polarizability which are modelled by equation (2) and (3).

$$E = E_o cos2\pi v_o t \quad (2)$$

$$\alpha = \alpha_o + \left(\frac{\partial \alpha}{\partial q}\right)_0 q_o + \cdots \quad (3)$$

where q_o is the vibration amplitude of nuclear displacement, q which is the vibrating molecules with a vibration frequency, v_m.

$$q = q_o cos2\pi v_m t \quad (4)$$

Substituting equation (2), (3) and (4) into (1) produces,

$$P = \alpha_o E_o cos2\pi v_o t + \frac{1}{2}\left(\frac{\partial \alpha}{\partial q}\right)[\cos\{2\pi(v_o + v_m)t\} + \cos\{2\pi(v_o - v_m)t\} \quad (5)$$

The first term in equation (5) represents the Rayleigh scattering, the second term is the Raman scattering with two radiation frequencies, $v_o + v_m$ (anti-stokes) and $v_o - v_m$.(stokes). Equation (5) shows that for a molecule to be Raman active, the rate of change of polarizability, $\frac{\partial \alpha}{\partial q}$ must not be zero[14].

The amount of inelastic scattering is infinitesimally small relative to that of 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 [15]. SERS amplifies the Raman signal by binding the molecules with noble metal, known as substrate. Nano-particles (MNPs) gold, silver and copper are common examples of substrate in use. The distribution of MNPs produces 'hot spot' which has higher strength of electromagnetic field. Interaction between molecule and MNPs 'hotspot' increases Raman scattering and thus effectively amplifies its intensity[16].

III. NON-STRUCTURAL PROTEIN 1

Virus of *Flaviviridae (genus Flavivirus*) is found to attribute to Japanese encephalitis, Murray Valley encephalitis, Tick-borne encephalitis, West Nile encephalitis, dengue fever, and yellow fever. *Flavivirus* genome is a single strand of ribonucleic acid (RNA). It consists of three structural proteins and seven nonstructural proteins[1]. Non-structural proteins are proteins encoded by a virus but it is not part of the viral particle. It forms in the host cell after the infection. Amongst the non-structural proteins, NS1 protein is the most important, with its role as alternative marker for viral diseases. It is believed to be responsible for virus replication process [17]. This protein acts as the antigen to stimulate antibody production in the immune system. The detection of antigen is prior to the detection of antibody which will allow early detection of diseases. Normally, ELISA protocol is used in the detection of antigen from serum, with problems associated with blood sampling.

IV. SALIVA

Saliva has become the most favoured alternative to blood as diagnostic fluid due to its advantages in sample collection procedure: non-invasive, simple and quantitatively feasible. Saliva samples also can be collected anywhere, with or without trained personnel. Other body fluids such as tears and sweat are difficult to obtain in sufficient quantity for routine test. While urine sample need to be collected in a private environment. As for blood, invasive collection, necessity for trained personnel and special equipment are always the issues.

Saliva can easily be obtained from oral cavity. It contains various compound: inorganic, organic, non-protein, protein, hormone and lipid molecules[18]. Saliva is produced by the salivary glands; parotid, submandibular, sublingual and minor salivary glands. For diagnostic application, gland-specific saliva or whole saliva are used as the analysis sample. Gland-specific saliva sample is normally used to understand gland-specific pathology while whole saliva sample is used for evaluation or systemic disorder. Whole saliva is a mixture of fluid containing secretion from the salivary glands, gingival crevicular fluid, nasal secretion, serum and blood derivatives, bacteria, viruses and food debris. Whole saliva can be collected using unstimulated and stimulated method [19]. As for SERS analysis, the most common saliva collection method used is unstimulated collection, by draining or spitting.

V. METHODOLOGY

Saliva samples were collected from volunteers aged 23-34 years old. All sample was collected in the morning (9-11am), abiding a published protocol [20]. The subject was also advised to refrain from taking foods, drink (except plain water) and brushing teeth at least 1 hour prior to saliva collection. About 10 minutes prior to the collection, subject was asked to gargle thoroughly for 1 minute. Thereafter, whole saliva of approximately 3ml was collected using unstimulated collection procedure. The sample was then transferred into Eppendorf tubes and was centrifuged at 14000rpm for 10 minutes to extract the supernatant.

NS1 glycoprotein protein (ab64456), expressed from E-coli, is from Abcam. It comes in a liquid form with concentration at 1mg/ml, equivalent to 1000ppm. NS1 of 10µL at 1000ppm was mixed with different amount of saliva supernatant to produce different concentration of NS1-saliva mixture: 500ppm, 200ppm, 100ppm, 50ppm, 25ppm, 20ppm, 12.5ppm and 10ppm. The mixture was then centrifuged at 5000ppm for 1min to ensure its homogeneity

NS1 of 10µL at 1000ppm, saliva supernatant and NS1-saliva mixture at different concentrations were deposited onto the GS substrate using micropipette. GS substrate is from Tedpella. Its supporting base plane is a high quality soda-lime glass standard microscope slide. The slide is coated with gold nanoparticles (Au). The gold surface is not atomically flat, but is corrugated in the nm range. The gold slides are auto-clavable.

The samples are then left to dry prior to Raman analysis. Raman spectra for this study were recorded by Perkin Elmer Raman Station 400F dispersive Raman spectrometer. The

excitation source is near infrared 785nm laser, with a minimum laser spot size of 100 micron. For all the spectra, the equipment was set to 10% of the full power, approximately 10mW on the sample. A high sensitivity open electrode CCD detector is employed. For better quality spectra, cosmic ray removal mode with median filter and baseline correction mode is enabled. All the spectra shown below are the best spectrum obtained from 3x3 point maps, with each point collected using a 5 second acquisition time unless specified otherwise.

VI. Result and Discussion

A. Surface Morphology and Raman Spectra of Gold Coated Slide Substrate

FESEM and AFM analysis were conducted to observe the surface morphology of the substrate[21]. Figure 2(a) and (b) show the 3D AFM image and FESEM image, respectively. Dense nano-size structure is observed from the images and its size is ranging from 40nm to 100nm.

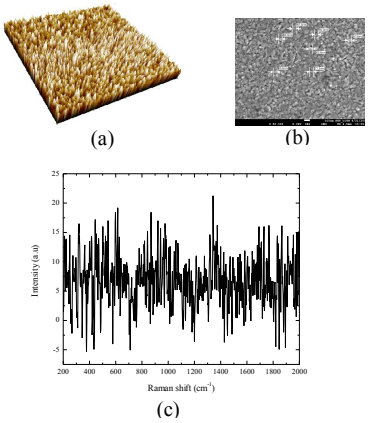

Figure 1: (a) AFM image of GS substrate, (b) FESEM image of GS substrate, (c) Raman spectrum of GS substrate

Figure 4(a) shows the average Raman spectrum of blank GS substrate. The maximum, minimum and average of peak intensity recorded are 21.24, -5.33 and 6.79a.u., respectively. For a peak to be distinct, a minimum intensity value of 30a.u. is necessary, anything less will be veiled by the background signal.

B. NS1 protein Characteristic Peak

Figure 3 shows the Raman spectrum of NS1 protein at 1000ppm. Peaks can be observed from the spectrum at 548cm^{-1}, 1012cm^{-1}, 1178cm^{-1}, 1540cm^{-1} and 1650cm^{-1}. Every peak is related to different vibration mode of the NS1 molecules. Since Raman spectrum of NS1 is non-existence in commercial standard spectra library, we select peak at 1012cm^{-1} to be the characteristic peak to fingerprint the NS1 protein. Reasons being it exhibits the highest intensity of approximately 1000a.u. and it is still observable at low concentration of NS1 (see Figure 4). Moreover, it is found to be attributed to ring breathing vibration of the trigonal ring of benzene ring structure, thus confirming the presence of benzene-ring in the structure of NS1 side chain molecule[22].

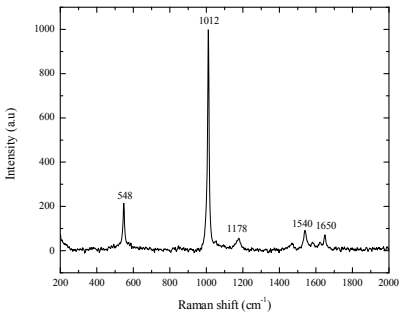

Figure 2: Raman spectrum of NS1 protein (1000ppm) on GS substrate.

C. Raman spectrum of NS1-Saliva Mixture

Raman analysis of NS1 at different concentration of NS1-saliva mixtures and saliva supernatant was conducted. Figure 4 shows spectra obtained from one of the subjects, others exhibit similar feature.

Spectra of blank GS and saliva in Figure 4(a) and (b) display no prominent peaks, showing they are Raman inactive. Spectrum for blank GS has been discussed in Section VI(A). As for the saliva supernatant, the spectrum is as expected since 94%-99% of saliva is water [23] which is a well-known Raman inactive molecule [14].

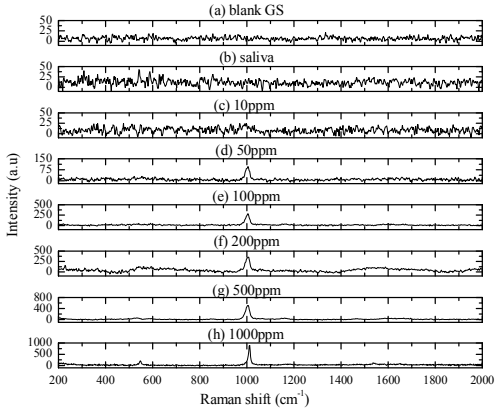

Figure 3: (a) blank GS, (b) saliva, (c)-(g) NS1-Saliva Mixture at different concentration spectra, (h) NS1

The peak at 1012cm^{-1} is found to be the only peak that is visible, even up to 10ppm. Other peaks present in the 1000ppm spectrum disappear as concentration decreases.

As concentration of NS1-saliva mixture decreases, the intensity of the peak follows, from Figure 4(h)–(c), similar to finding in our previous work in melamine [24]. For a low concentration mixture at 10ppm, the peak intensity remains, at 24.7a.u.

For further investigation into the relationship, characterization graph in Figure 5 is produced. A correlation coefficient (R) of +0.96118 is obtained, strongly suggesting a linear relationship between the concentration and characteristic peak intensity of NS1. A standard error estimation of 0.11382 at the estimated slope is obtained, showing small variation from the slope of regression line for different concentration of NS1.

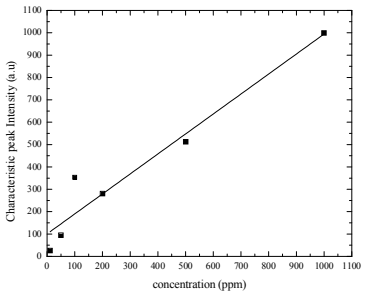

Figure 4: Characterisation of NS1in saliva versus concentration

VII. Conclusion

Our work finds NS1 is Raman active while saliva inactive. The SERS spectrum of NS1 with GS shows several peaks, with the molecular fingerprint marked as the distinct peak at Raman shift of 1012cm^{-1}. This is because the peak reflects breathing vibration of the benzene ring structure in the NS1 side chain molecule and exhibits the highest intensity, even up to 10ppm. So far, SERS spectrum of NS1 with GS is not found in any commercial standard spectral library. Additional experimental work on NS1-saliva mixture discovers the intensity of NS1 characteristic peak to decrease with concentration, with a correlation coefficient of +0.96118 and a standard error estimation of 0.11382. This linear relationship further suggests SERS being suitable for detection of NS1 in NS1-saliva mixture.

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