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Label-free surface-enhanced Raman spectroscopic detection of HIV-1 infection in blood and plasma adsorbed on conductive silver pasted glass substrate

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This study reports on application of conductive silver paste smeared glass slides as Raman spectroscopy sample substrates for label-free detection of HIV-1 p24 antigen in blood plasma. It showed that the same substrates can be applied in Raman spectroscopic screening of blood plasma for presence of HIV. The characteristic Raman spectrum of HIV-1 p24 antigen displayed prominent bands that were assigned to Ribonucleic acids (RNA) and proteins that constitute the antigen. These spectra can be used as reference during Raman spectroscopic screening for HIV in plasma within the first few days after exposure (<7 days). The Raman spectra obtained from HIV+ plasma displayed unique peaks centered at wavenumbers 1270 and1446 cm^{-1} attributed to the Raman active vibrations in the virion proteins. Other bands similar to those reported in literature were also seen and assigned to lipids and carbohydrates. The attachment of the HIV virions to silver nanoparticles via gp120 glycoprotein knobs were thought to be responsible for the enhanced Raman signals of proteins associated with the virus. The principal component analysis (PCA) applied on the combined spectral data showed that HIV- and HIV+ spectra had differing spectral patterns. This indicated the great power of Raman spectroscopy in HIV detection when plasma samples are deposited onto silver paste smeared glass substrates. The Raman peaks responsible for the segregation of the spectral data in PCA were mainly those assigned to the viral proteins. Excellent results were also obtained from Artificial Neural Network (ANN) applied on the HIV+ data with R (coefficient of correlation) and R^2 (coefficient of determination) values of 0.9958 and 0.9895, respectively. The method has the potential of being used as quick blood screening for HIV before blood transfusion with the Raman peaks assigned to the virion proteins acting as a reference.

Key words: Raman spectroscopy, HIV-1 p24 antigen, HIV+ and HIV-, PCA and ANN

INTRODUCTION

In the early years of Acquired Immunodeficiency Syndrome (AIDS) history, Human Immunodeficiency Virus (HIV) was unknown, misunderstood, feared, untreatable, fetal, dreaded by many and often linked to

traditions (Coffin et al., 1996). With the aim of understanding and eventually developing a cure for AIDS, HIV became the most intensively studied virus in human history (Wu and Zaman, 2012). Great progress has been made in obtaining an outline sketch of how genes and proteins in HIV particles operate and in understanding the biochemical specificity of HIV (Greene and Warner, 1993), the factors controlling its replication, the pathology of how it destroys the human immune system and the molecular bases of HIV infection and immunosuppression (Pantaleo et al., 1993). There have been several attempts of developing new HIV detection techniques and most of them are yet to be available commercially. Some of the methods have aimed at detecting the HIV particles which include nanospectroscopic assays (Block et al., 2012), photonic crystal biosensors (Shafiee et al., 2014), electrical sensors (Shafiee et al*.,* 2015) and surface-enhanced Raman spectroscopy (Lee-Ho et al., 2015; Lee et al., 2015). Those that were designed to detect the HIV-1 p24 antigen includes electrochemical immunosensors (Gan et al., 2013), electro-chemiluminescence immuno sensor (Zhou et al., 2015), X-Ray Diffraction (XRD) methods (Sun et al*.,* 2005) and Near Infrared (NIR) spectroscopy (Sakudo et al., 2005; Rahim et al*.,* 2010). According to the UNAIDS 2016 report, by year 2015, 36.7 million people globally were living with the human immuno-deficiency virus (HIV), 2.1 million new infections and 1.1 million deaths were reported (UNAIDS, 2016). Till date there is no known cure for HIV but its transmission and progression is checked through administration of highly effective antiretroviral therapy (ART) (Bloch et al., 2012).

The widely used method for the detection of HIV in blood and recommended by World Health Organization (WHO) is based on the presence of specific antibodies associated with the virus (Ezzel, 2002). This method of diagnosis does not detect directly the presence of viral antigen or Ribonucleic acid (RNA) associated with the virus. Further, the method only works after about 90 days from initial exposure to the virus since specific antibodies are not produced before then (Moor et al*.,* 2013). Unfortunately, this is the only method accessed by the majority of diagnostic facilities in developing nations. At the early stage of HIV infection, the generic symptoms are often difficult to distinguish from those associated with common ailments such as influenza or fevers. Such symptoms include; sore throat, chronic diarrhoea, skin rushes, swollen glands, sweats especially at night and nausea (Wu and Zaman, 2012).

There are many advantages of making early HIV detection and includes reducing anxiety of those suspecting to have been exposed, to get a better sense of how and when one got exposed, get early access to support services and prevent new infections. Due to the large extent of the HIV epidemic and the ever high new infections, a number of assays have been developed and new ones explored for detection. The methods in use currently include enzyme-linked immunosorbent assay (ELISA), polymerase chain reaction (PCR) and western blot among others (Shafiee et al., 2015). These techniques, though reliable and specific, suffer from many disadvantages such as being time consuming, expensive and unsuitable for large scale routine screening and covers a narrow range of HIV antigen concentration (Lee et al., 2015). Besides, most of these techniques are unreliable when used within the window period (between 7-90 days after exposure), although a combination of ELISA and PCR has shown to be able to detect HIV-1 p24 antigen as early as 7 days after exposure (Barletta et al., 2004).

Raman spectroscopy is a technique based on monochromatic light scattering. It provides key information on the structure of molecules based on the fact that no two molecules can give exactly the same Raman spectrum (Banwell and McCash, 2007). Different molecules have different vibrations; thus the Raman spectrum of molecules are unique making identification easier (Moor et al*.,* 2013). The position and intensity of features in the Raman shift spectrum is used to study molecular structure and to determine the chemical identity of the sample (Pascut et al., 2011). This technique has been explored as a fast, sensitive, selective, real time detection and reliable screening method for detection of various infections. However, there are still some limitations associated with Bulk Raman Spectroscopic technique that hinders further clinical applications in medical diagnosis. A major limitation being the efficiency of Raman scattering is extremely weak for biological samples (Lin et al*.*, 2011). There is a need to increase the laser power as well as exposure time to acquire good quality spectra, which may change and even damage the biological sample (Vo-Dinh et al., 2005). The development of surface-enhanced Raman spectroscopy (SERS) based on nanotechnology has been used to overcome the drawbacks associated with Bulk Raman Spectroscopy. In addition to signal enhancement, SERS can provide further advantages over other spectroscopic techniques:

(1) Work well with aqueous solution since it does not suffer from absorption as in the case of IR;
(2) No sample preparation;
(3) Wide concentration ranges;
(4) Sensitive and specific tool to different molecules;

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(5) Not affected by temperature much and
(6) Offers non-destructive analysis (Lin et al*.,* 2011).

SERS has recently been employed in detecting blood and plasma fingerprints for gastric cancer (Feng et al*.,* 2011), medical diagnostic and biological imaging (Vo-Dinh et al*.,* 2005), colorectal cancer (Lin et al*.,* 2011), cervical cancer (Feng et al., 2013) and nasopharyngeal cancer (Lin et al., 2014; Feng et al., 2010; Lin et al., 2010). The capability of SERS to distinguish between blood and plasma components from healthy volunteers and patients based on individual blood and plasma components such as DNA/RNA, lipids, carbohydrates and proteins have also been demonstrated with well-defined tentative peak assignment (Feng et al., 2011; Lin et al., 2014; Notigher et al., 2004). Rapid and sensitive determination of HIV-1 based on SERS was first reported by Lee et al. (2015); however, in their study, measurements were done on HIV-1 virus-like-particles (HIV-1 VLPs). In this study, blood and plasma characterization is based on real HIV-1 infection.

In this research study, detection of HIV-1 in whole blood and blood plasma pipetted on silver painted microscope glass slide whose nanoparticles is used as simple and cheaper SERS substrate was investigated. Silver substrate has been used in a number of SERS based research using a complex predetermined mechanism resulting in regular nanoparticle. Through painting a glass slide using silver air dying paint, a considerable enhancement was equally able to be achieved in not only a simple but also a cheaper way.

MATERIALS AND METHODS

Sample collection

Blood and its corresponding plasma samples were obtained from 68 volunteers (40 patients and 28 healthy individuals) after a consented agreement. All volunteers provided written informed consent to participate. This study was approved by the Kenyatta National Hospital-University of Nairobi (KNH-UoN) Ethics and Research Committee (Proposal number: P637/10/2015). Samples were collected after overnight fasting in Ethylene Diamine Tetra Acetic acid (EDTA) collection tubes. After collection of blood, PCR test was performed on the blood samples to classify them as either HIV-1 positive or negative samples. The samples were then temporarily stored in a vertical rest position at -200°C in the laboratories of the University of Nairobi institute of Tropical and Infectious Diseases (UNITID). During this period, blood and plasma were separated based on their densities, with plasma (a pale yellow fluid) occupying the upper part being less dense. The plasma was then pipetted out into a different EDTA container. All samples were then transported to Laser lab for Raman spectral fingerprinting.

SERS substrates preparation

The glass slides were cleaned using ethanol and then air dried for about 20 minutes. The silver conductive paste (SPI suppliers, USA) was smeared using a small brush onto a microscope glass slide and air dried for about an hour. The silver paste consisted of mixture of silver metal particles (35-65% of total weight), 1-methoxy-2 propanol acetate (10-30% weight), butyl acetate (10-30% weight) and acrylic resin (5-10% weight). The procedure was repeated till the silver colloids and silver deposition over a deposition surface appeared as nanospheres. This preparation left the glass slide surface with nanoparticles or aggregates of particles that are capable of serving as metal roughness features as shown in Figure 1a and 1b under x10 objective lens (owing to its spectral lower signal to noise ratio as compared to other objective lenses). On different Raman substrate prepared, HIV-1 p24 antigen stored in Roswell Park Memorial Institute (RPMI) 1640 medium, blood and plasma samples were smeared on the surface and the adsorbate left to dry for about 1 h 30 min before taking Raman measurements.

SERS measurement

Raman spectra were obtained using a Laser Confocal Raman microscope (STR Raman Spectrum System, Seki Technotron Corp; Model number RO-110J) with laser excitation wavelength of 785 nm and a laser power of 150 mW. Raman spectra were recorded by focusing the 10% laser power directly on the dried smears of blood and plasma samples on the SERS substrate to reduce photo degradation that may occur when the laser power is higher. The laser was focused on the samples with x10 objective lens. Raman scattered light from the sample was collected using the same objective lens and detected by a Charge Coupled Device (Princeton Instruments; Acton SP2300) equipped with a 256 x 1024 pixel camera cooled at -76°C. The excitation parameters were; diameter of laser spot at the focus point ≈ 71 µm, excitation power was 150 mW, along with exposure time of 15 s and 15 accumulations per spectra. The grating chosen in order to cover a wider spectral range was 600 lines per mm grating. Background spectra were obtained by blocking the laser source from being scattered by the sample. The Raman equipment was calibrated daily using the 520.5 cm^{-1} band of a silicon wafer. For both the control and the infected samples, spectra were recorded from 10 different spot areas. Raman spectra were recorded in the spectral range 157–1800 cm^{-1} with center wave number of 1100 cm^{-1}. The raw Raman spectral data from each of the samples were first smoothened using Savitzky-Golay filtering function (at 9 points, second derivative) in MATLAB, and autofluorescence background removed using a Vancouver Raman Algorithm based on fifth-order polynomial fitting method developed by Zhao et al. (2015). Multivariate statistical analysis was done using PCA and ANN to differentiate between Raman spectral data profiles from plasma with HIV-1 and from those without.

RESULTS AND DISCUSSION

Characterization of plasma samples

Averaged spectra of HIV- plasma, HIV+ plasma (at selected viral loads; red line - 193, blue – 2753, green – 21771, purple – 37368 and grey – 835020 copies per ml of plasma) and silver paint smeared glass substrate within the spectral finger print region 350-1750 cm^{-1} are displayed in Figure 2 and their tentative peak assignment in Table 1. The peaks in the spectra are associated with lipids (713, 928, 1446 cm^{-1}), proteins (645, 713, 813, 1270, 1446, 1658 cm^{-1}) and carbohydrates (928, 990 cm^{-1}) (Virkler and Lednev, 2009; Feng et al*.,* 2010*,* 2011, 2013; Lin et al*.,* 2012). Most Raman bands in HIV+

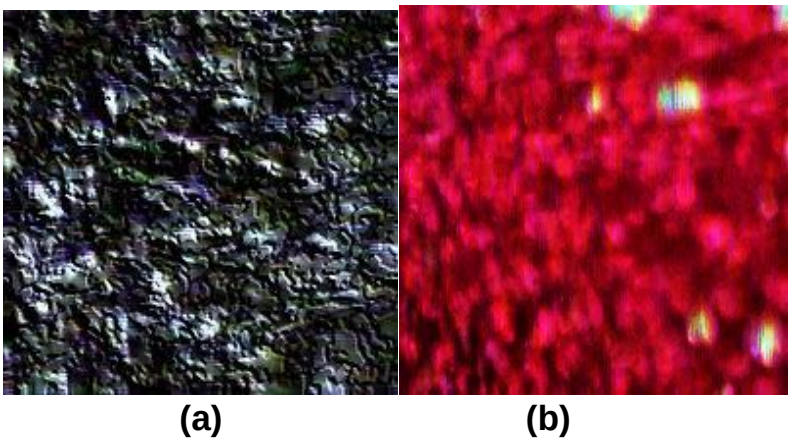

Figure 1. (a) Ag substrate (b) Blood adsorbed on Ag substrate.

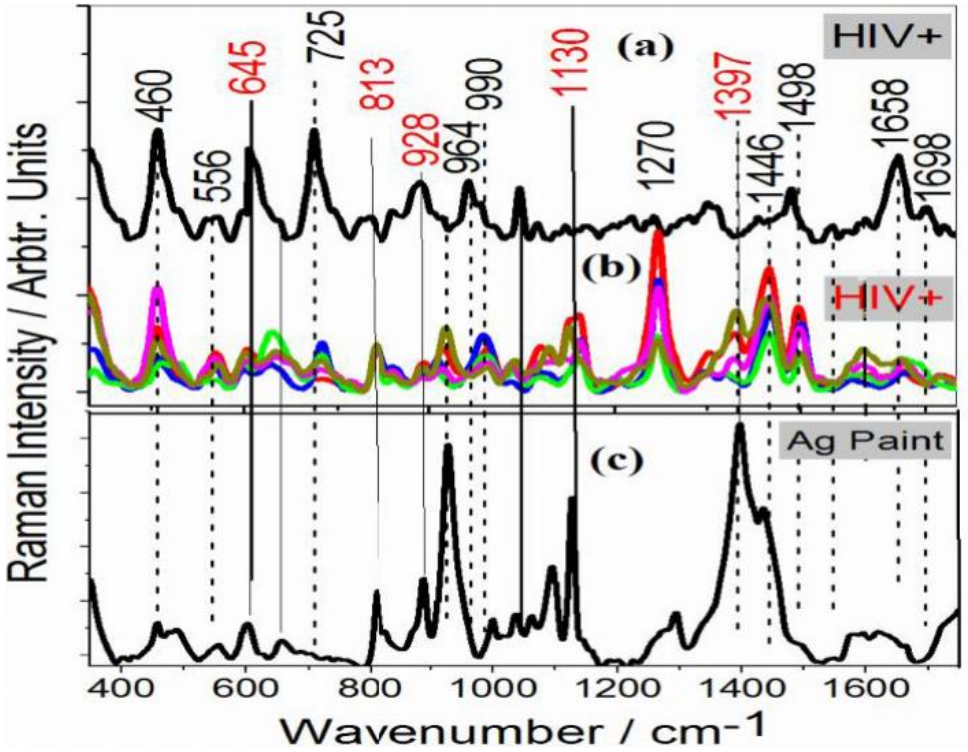

Figure 2. Raman Spectral Profile of HIV- Plasma (a), HIV+ plasma (b) after baseline correction and Silver substrate (c) –inset. HIV- spectra was vertically shifted for clarity.

plasma were similar to HIV- bands; however, HIV+ displayed unique peaks centered at wavenumbers 1270 and 1446 cm^{-1}. These peaks were assigned to Raman active vibrations in the HIV virion proteins (Virkler and Lednev, 2009; Zinin et al*.,* 2011; Lee-Ho et al., 2015).

The presence of silver nano-particles on the substrate, though sparse (35-65% per weight in the paste), are thought to cause the HIV virus particles to get attached to it resulting in the strong Raman signals of associated proteins. Reportedly, the HIV virus has a preferential attachment with the silver nano-particles via gp120 glycoprotein knobs (Elechiguerra et al., 2005). The two intense peaks centered at wavenumbers 1270 cm^{-1} and at 1446 cm^{-1} were ascribed to vibrational state of amide III of α-helix and CH_2 bending vibrational mode in proteins and lipids components of plasma respectively (Feng et al*.,* 2010*,* 2011*,* 2013; Lin et al*.,* 2012). These results were similar to those reported by Xu and Lu (2005)

Table 1. SERS peak positions and vibrational mode assignments.

HIV- (cm^{-1})	HIV+ (cm^{-1})	Band Assignment	Vibrational mode
460	460	Unknown	Unknown
556	556	Unknown	Unknown
638	638	*v*(C-S)	L-tyrosine (Protein)
713	Missing	*v*(C-S) and *vs* (C-N)	Proteins; Lipids
725	725	Unknown	Adenine (RNA)
813	813	Unknown	RNA; Alanine
928	928	*δ*(COH) and *v*(C-C)	Carbohydrates; Lipids
964	Missing	Unknown	Unknown
990	990	CH_2 Rocking	Carbohydrates
1206	1206	Ring Vibration	Tyrosine, phenylalanine
Missing	1270	*δ*(CH_2)	Amide III (Proteins)
Missing	1446	*v*(CH_2), *δ* (CH_2) and *v*(CH)	Proteins and Lipids
1498	1498	*vs* (NH_3)	Spermine Phosphate hexahydrate
1658	Missing	*v*(C=O)	Amide I
1698	Missing	Unknown	Unknown
v -Stretching	Vibration	*δ* – Bending Vibration	*vs* - symmetric stretch

Source: Feng et al. (2010, 2011, 2013); Lin et al. (2012).

from serum samples (plasma without clotting factor) showing that using the cheaper silver paste smeared glass substrates works equally good.

Principal component analysis of the HIV-1 p24 contaminated and uncontaminated plasma Raman spectral data

The great potential of Raman spectroscopy for use in HIV-1 screening within the window period (<14 days after exposure) was demonstrated when used with PCA on the Raman data set obtained from human plasma intentionally contaminated with HIV1-P24 antigen and from uncontaminated plasma (Figure 3). The first two PCs (PC1 and PC2) accounted for 96.69% of the total variance of the original matrix.

Plasma samples contaminated with HIV1-p24 antigen could be clearly distinguished from those without the antigen with sensitivity of 96.5% (28/29) and specificity of 91% (10/11). This indicated that the two Raman spectral datasets had differing spectral patterns thus demonstrating great power of Raman spectroscopy together with PCA in early screening for HIV-1. The samples containing HIV1-p24 antigen had mainly negative PC2 scores while those without the antigen had positive PC2 scores in the score plot.

Segregation of HIV- from HIV+ Raman dataset plasma samples using principal component analysis

PCA algorithm was also run on the data matrix after data preprocessing as shown in Figure 4. With PCA, even the subtle spectral differences in the data sets were utilized in the differentiation. It was found that the first two PCs accounted for 99.76% of the total variance with PC1 (99.03% variance) and PC2 (0.73% variance) of the original plasma data matrix as shown.

Score plots of PC1 and PC2 were able to highly distinguish the spectra of plasma from infected group (n=48) and control group (n=28). HIV- plasma samples could be clearly distinguished from those obtained from infected volunteers without using any labeling probe. This was achieved with sensitivity of 100% (48/48) and specificity of 89.28% (25/28).

Principal component analysis of blood samples

The potential of chemometric SERS for detection of HIV-1 infection in blood was also demonstrated with the application of PCA on the Raman data set obtained from human blood infected with HIV-1 (n=18) and from healthy blood samples (n=18). PCA algorithm was run on the data matrix after data preprocessing. It was found that the first two PCs accounted for 99.89% (PC1 = 99.64% and PC2 = 0.25%) of the total variance of the original data matrix (Table 5).

Score plots of PC1 and PC2 was highly distinguishing for the spectra of infected group and control group. As can be seen in Figure 5, blood samples from healthy volunteers could be clearly distinguished from those obtained from infected volunteers without using any labeling probe. This was achieved with sensitivity of 83.3% (15/18) and specificity of 100% (18/18) indicating

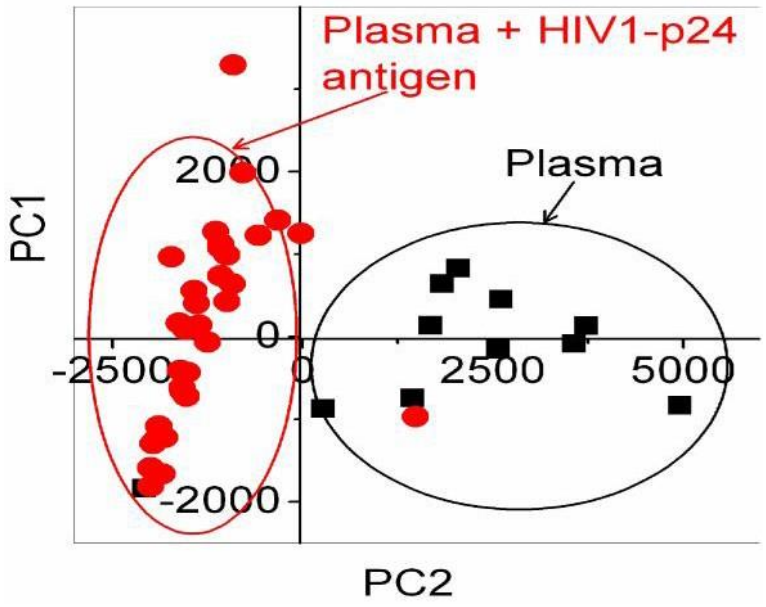

Figure 3. Plots of the first principal component (PC 1) versus the second principal component (PC 2) for spectral data obtained from negative plasma and plasma contaminated with p24 antigen.

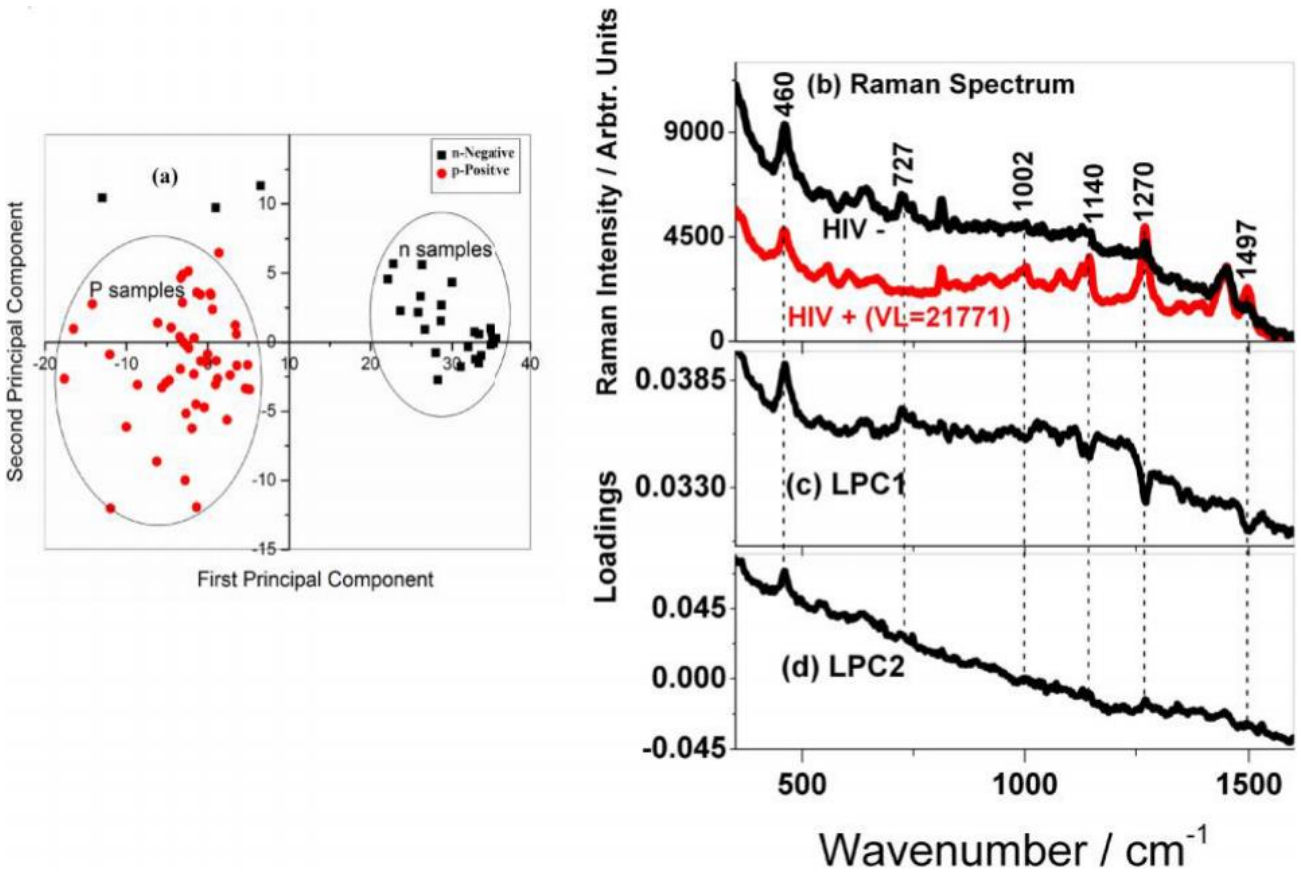

Figure 4. Plots of the first principal component (PC 1) versus the second principal component (PC 2) for healthy group and HIV-1 infected group plasma samples (a), Selected Raman spectrum of HIV- (black line) and HIV+ plasma (red line) (b), Loading plots of PC 1 (c) and Loading plots of PC 2 (d).

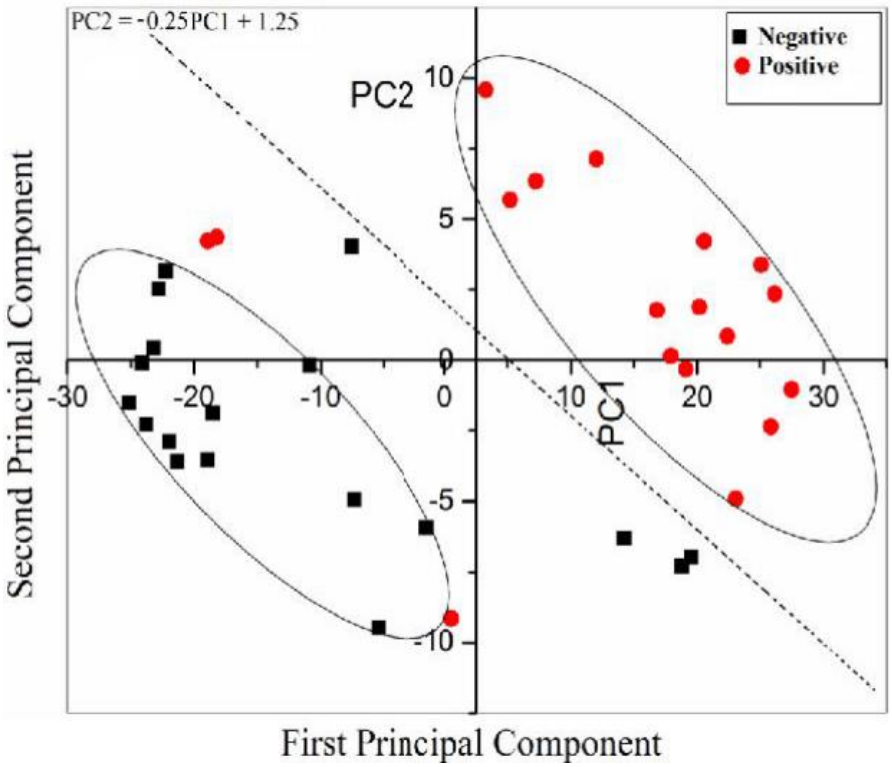

Figure 5. Plots of the first principal component (PC1) versus the second principal component (PC2) for healthy group (black shaded squares) and infected group blood samples (red shaded circles).

the applicability of Raman spectroscopy together with PCA in label free detection of HIV-1 infection. Majority of infected samples had mainly positive PC1 scores while the control samples had mainly negative PC1 scores in the score plot.

Prediction of HIV-1 infection using Artificial Neural Network

An ANN technique was also evaluated to predict HIV-1 infection from SERS dataset of both infected and control whole blood as well as their corresponding plasma Raman datasets. When presented with the testing set, the network generated an output with correlation coefficient value in the range of 0.0 (0%) ≤ R≤1.0 (100%) reflecting its predictive value for HIV-1 infection. R^2 can be defined as the ration of explained variation to the total variation of the matrix dataset (Floyd and Tourassi, 1992). 0% indicates that the model explains none of the variability of the response data around its mean while 100% indicates that the model explains all the variability of the response data around its mean. The ANN training and testing model resulted in R^2 = 0.99994 for HIV+ plasma sample and R^2 = 0.9601 for HIV+ whole blood samples indicating that the detection achieved clinically relevant precision (Figures 6 and 7). Generally, R^2 values higher than 0.9 indicate that the method under investigation is clinically accurate (Enejder et al., 2002). Based on this criterion, our method of HIV detection was clinically accurate.

However, when a network trained using infected Raman dataset was used to test a control Raman dataset of the same fluid type as a target, the value of square of correlation coefficient from the trained network was approximately equal to zero. Similarly, when a network trained using control Raman dataset was used to test an infected Raman dataset of the same fluid type as a target, the value of correlation coefficient from the trained network resulted in $R^2 \approx 0$ (Figure 8).

Conclusion

In summary, SERS was applied to determine the effect of HIV-1 infection on blood and plasma components. Raman spectral profile of the infected samples showed specific biomolecular information including reduction in body components especially proteins and lipids as compared to the healthy samples. Therefore, SERS may be a suitable candidate for evaluating HIV-1 infection related changes in blood and plasma samples thus

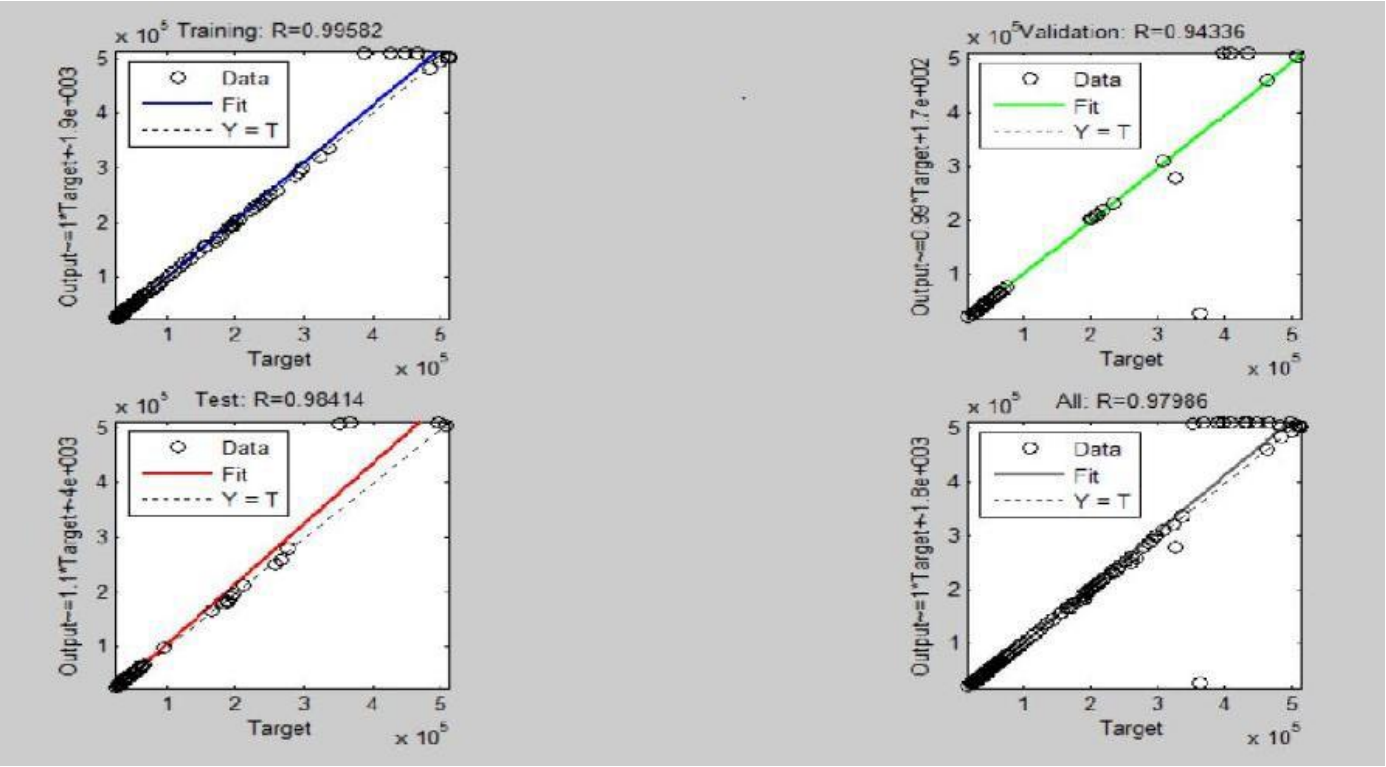

Figure 6. ANN correlation output from HIV+ whole blood Raman dataset. Training and testing dataset from HIV+ whole blood achieved a diagnostic regression R2 = 0.9601 (R = 0.97986).

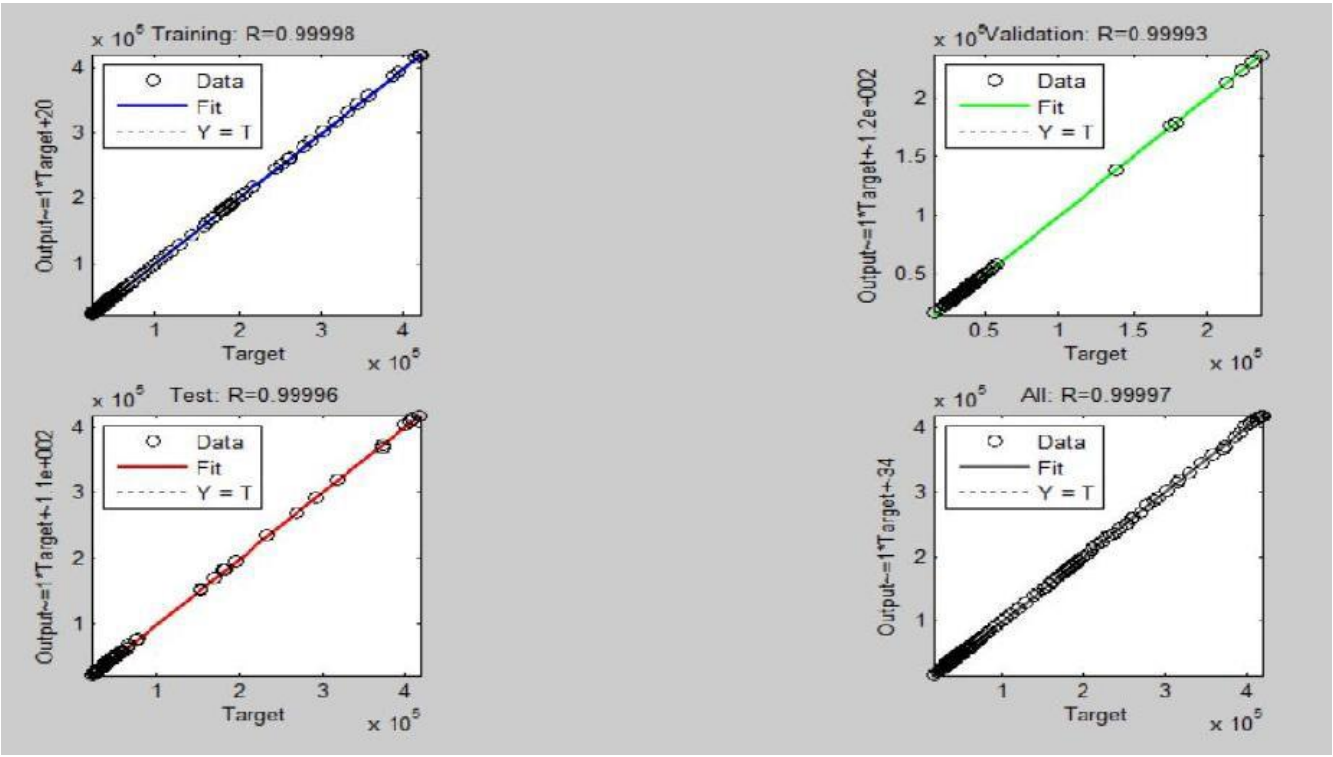

Figure 7. ANN correlation output from HIV+ plasma Raman dataset. Training and testing dataset from HIV+ plasma achieved a diagnostic regression R2 = 0.99994 (R = 0.99997).

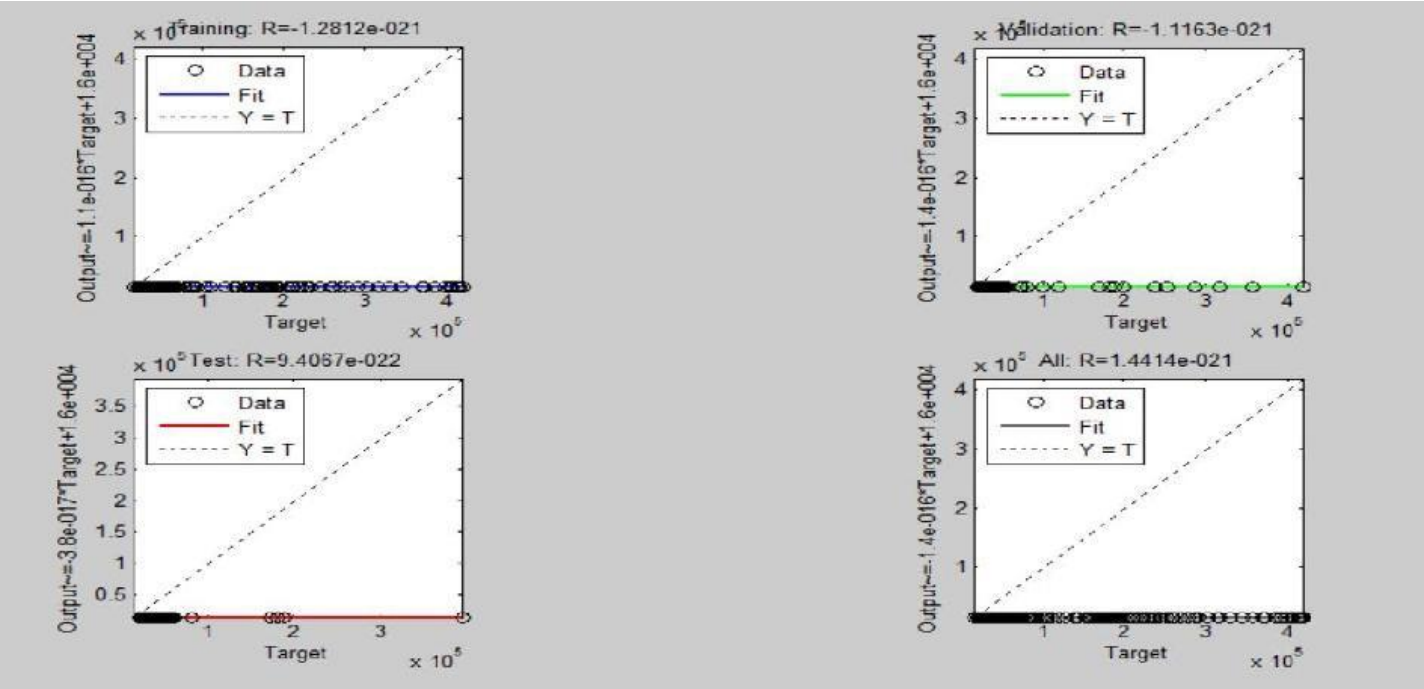

Figure 8. ANN correlation output trained using infected plasma Raman dataset but tested on control plasma Raman dataset.

providing useful information that can really help in diagnosis especially at individual level and potentially early screening of the virus.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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