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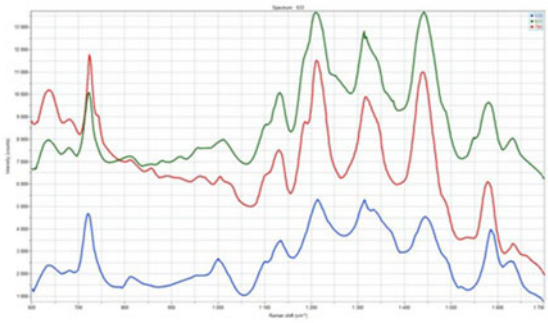

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Research on Measurement Conditions for Obtaining Significant, Stable, and Repeatable SERS Signal of Human Blood Serum

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Abstract: The Raman spectra of human blood serum can be used to identify cancer or other diseases; however, obtaining a reliable surface enhanced Raman scattering (SERS) signal of human blood serum is difficult. Two primary factors that affect SERS measurement of serum are photodegradation and sample composition, which are investigated in this research. In the end, this research proposes a promising set of measurement conditions that can both acquire reliable serum Raman signals and avoid photodegradation.

Index Terms: Raman spectroscopy, blood monitoring, optical diagnostics for medicine, optical standards and testing.

1. Introduction

Raman spectroscopy is a well-established analytical technique based on the inelastic scattering of monochromatic light by the vibrating atoms constituting the sample. It is widely used in fields such as materials, food, medicine, chemistry, and so on [1]–[5].

One of Raman spectroscopy's main disadvantages is its relatively weak signal strength. The discovery of surface enhanced Raman scattering (SERS) can be used to overcome this disadvantage. SERS was discovered by Fleischmann *et al.* in 1974 and later confirmed by Van Duyne *et al.* in 1977 [6]–[8]. SERS can improve the weak Raman scattering signals; its enhancement factor can be as high as 10^{14} and can be used to detect trace substances [4], [8]–[10].

For SERS to take effect, the sample molecules must be adsorbed on to the surface of metal nano-particles, usually silver (AgNPs) or gold nano-particles (AuNPs) [3], [4], [11]. Multiple metal nano-particles aggregated together can increase the SERS enhancement factors [3], [4], [11], [12], which is essential for the detection of trace substances. These structures that contain AgNPs or

AuNPs or so on and can enhance Raman signals are called SERS substrates. There are usually two types of SERS substrates: a) nano-particle colloidal made from Lee and Miesal or similar methods [13] and b) solid substrates that either come from deposition of nano-particle colloidal, or reduction of silver nitrate ($AgNO_3$) or chloroauric acid ($HAuCl_4$) [14]–[17]. Solid state substrates are used in this research because they have relatively high enhancement factor for serum, which contains large protein molecules [3], [5]. The solid substrates used in this research are AgNPs substrate produced by us using a modified version $AgNO_3$ reduction on TiO_2 method [12], [18]. This type of SERS substrates are used because they are simple and effective; they have good uniformity and film robustness [19], [20]; The performance of the substrates were tested using Rhodamin 6G (R6G): they can be used to detect 10^{-8}M R6G, and have an intensity standard deviation of less than 20%.

Using SERS to measure various body fluids is a method for the detection of various diseases [3], [21]. Particularly, in cancer detection, the existence of various cancer specific molecules, such as vascular endothelial growth factor (VEGF) or circulating tumor DNA (ctDNA) in body fluids may change blood or other body fluid's Raman spectra. This means Raman spectroscopy may be able to help determine early stage cancer in non-invasive medical examination and help with patient survival [22]–[24].

Currently, there are also SERS measurement technology that target specific biomarkers, most notably ctDNA and VEGF [25], [26]. These technologies may even help drug delivery or treatment of cancer [9], [27]–[30]. However, these methods often need labels that target the biomarkers, such as aptamers, and methods for producing aptamers are often complicated.

The body fluid used in this research is human blood serum. Different researcher teams use different measurement instruments, laser setups and sample preparation methods, which make their measurement results difficult to compare and some of the measurement results might be unreliable. Obtaining reliable, consistent and repeatable serum SERS signal is also difficult. As stated in the previous section, large protein molecules in serum may hamper the aggregation of nano-particles in metal nano-particles colloidal; serum is vulnerable to photo-degradation, which may cause abnormal Raman peaks that could interfere with serum signals. Also, due to relatively weak signal of serum and especially diluted serum, contamination on SERS substrate may interfere significantly with the serum's signal.

The goal of this research is to find a precise SERS measurement method, so that researchers can obtain consistent and repeatable serum Raman signals. This research focuses on solid substrates and mainly investigates two subjects: a) photo-degradation and methods to avoid it and b) the effect of serum sample composition and preparation.

2. Materials, Methods and Instrumentation

2.1 Materials

Human blood serum samples from healthy individuals and lung adenocarcinoma patients are provided by Zhejiang Cancer Hospital.

Analytically pure Tetrabutyl titanate, acetylacetonate and nitric silver and other lab reagents such as nitric acid and ethanol, as well as quartz slides are purchased from Hangzhou Mike Chemical Instruments Co., Ltd.

2.2 Ethics Statement

The individuals and patients have been informed and their written consents were obtained by Zhejiang Cancer Hospital.

The researches of the human samples in this manuscript are consistent with Declaration of Helsinki, and are approved by the ethics committees in Changchun Institute of Optics, Fine Mechanics and Physics of Chinese Academy of Sciences, China Jiliang University, and Zhejiang Cancer Hospital.

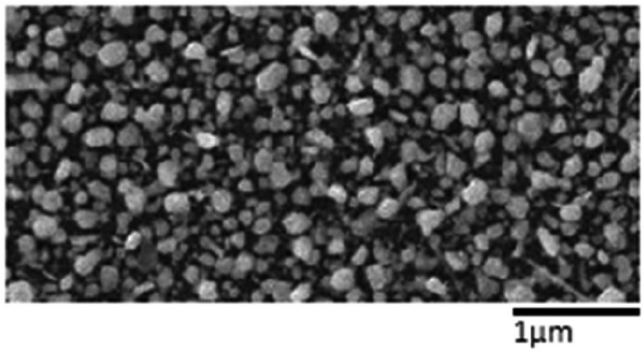

Fig. 1. Solid SERS substrates produced for this research, under SEM, measured using TESCAN VEGA3 SBH.

2.3 Methods for Making SERS Substrates

Method for making TiO_2 photo-catalyzed AgNPs substrates is modified from methods by Li *et al.* and Mazzocut *et al.* [12], [18].

In our method, TiO_2 colloidal is dip-coated on quartz slides, instead of glass slides as in Li *et al.* work, at a dip speed of 190 mm per minute; then, the coated slides are calcined in a muffle furnace at 510 degrees Celsius for 60 minutes; then, the substrate is immersed in $AgNO_3$ solution, and illuminated with filtered 254 nm UV light for 90 minutes, instead of the unfiltered 254 nm UV light as in Li *et al.* and Mazzocut *et al.* works.

Substrates are 5 $\times$ 5 mm and the SEM image of the SERS substrates is shown in Fig. 1. The substrates produced using this method was able to detect 10^{-8}M R6G, and the results have a standard deviation within 20%, which is an improvement over Li or Mazzocut's method.

2.4 Serum Processing

Different concentrations of serum are deposited on to the solid SERS substrates by a) dropping 15 μL of serum or diluted serum on the substrate or b) immersing the substrate in diluted serum for 30 minutes.

After samples are made, they are dried at 4 degrees Celsius in a refrigerator until dry. For serum dropped on the substrate, it takes four hours to dry; for serum deposited on substrate by immersion, it takes 30 minutes to dry.

2.5 Raman Measurement

Raman measurement is carried out using a Horiba LabRAM Evolution 800 Raman spectrometer; 50X long focal length objective was used; numerical aperture is 0.5, and working distance is 10.6 mm. Three laser modules are used: a 50 mW 532 nm laser, a 50 mW 633 nm laser and a 90 mW 785 nm laser. The focal point beam spot diameter for 532, 633 and 785 nm wavelengths are 1.298, 1.544, and 1.915 μm, respectively. Different laser power output, from 0.1% to 25% is monitored by using the neutral density (ND) filter within the Raman spectrometer. Maximum (100%) laser power for 532, 633 and 785 nm wavelengths at the focal point are 21.7, 24.8 and 53.4 mW, respectively.

In experiments for photo-degradation and laser set up, one spot on a substrate is measured once, then illuminated by laser of the same power for 60 seconds, and then measured again. The 60 second illumination in between measurements is used to photo-degrade the sample on purpose. The illumination and measurement procedure is repeated twice to get a total of three measurements.

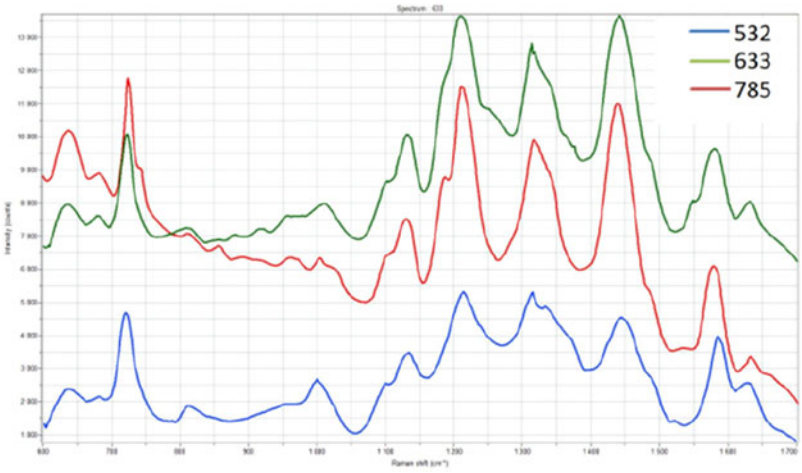

Fig. 2. Pre-baseline correction spectra using 532 (blue), 633 (green), and 785 nm (red) laser; all three spectra have clearly identifiable Raman peaks.

In experiments for comparing different serum compositions, the averages of 10 spectra collected from 10 different spots on the substrate are compared.

3. Results and Analysis

3.1 Fluorescence

While fluorescence is a problem often occurred in the Raman measurement of many types of biological or organic materials, it does not seem to have significant interference with the SERS measurement of serum. Fig. 2 shows Raman measurement of serum using 532, 633 and 785 nm laser, before baseline correction and smoothing. As shown in Fig. 2, in all three spectra, Raman peaks are easily visible.

3.2 Photo-Degradation and Laser Set-Up

The key to avoid photo-degradation is to reduce the optical power density on the substrate, and off-focus measurement is the primary method used in this research. When the laser is focused above the substrate surface, the light spot is larger and optical power density is reduced. On the other hand, Raman signal is not reduced as much.

In this experiment, the sample substrate is immersed in serum solution composed of 125 μl serum of one person and 375 μl normal saline (0.9% sodium chloride solution). Different laser wavelengths are used, and ND filters are used to modify laser power from 0.1% to 25%. Different off-focus distances: 10, 20, and 30 μm are used. Forty μm or above off-focus distances are not used because the Raman intensity decreased significantly: the light spot became too large for the instrument's confocal hole to capture the entirety of the whole light spot.

When 532 nm laser is used, it is difficult to avoid photo-degradation; as shown in Fig. 3, when laser is focused 30 μm above the surface, using 1% power of 532 nm laser (power density is 23 W/cm^2) produces very volatile Raman signals, which indicates that there is photo-degradation; as seen from Fig. 3(a), the three Raman spectra have different peak positions, band widths and intensities. Using 0.1% power of 532 laser (power density is 2.3 W/cm^2) still produces Raman signals that are inconsistent; as seen from Fig. 3(b), the consistency in peak positions and band widths improved, but the three spectra still show volatile peak intensities.

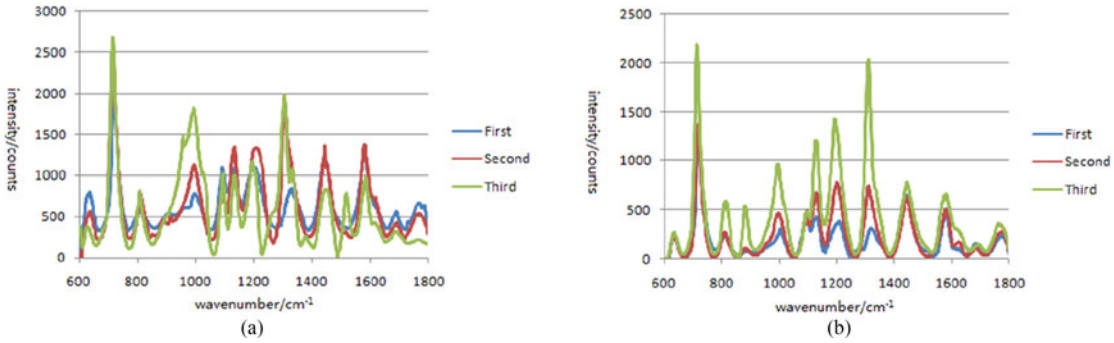

Fig. 3. The 532 nm laser measurements when laser is focused 30 μm above the surface. (a) uses ND filter that allows 0.1% laser power, while (b) uses ND filter that allows 1% laser power.

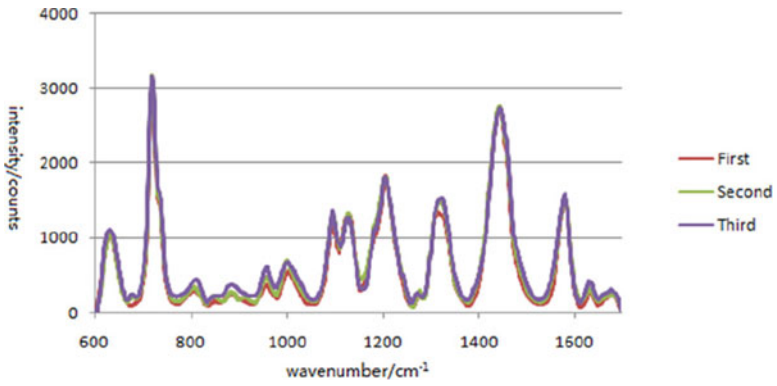

Fig. 4. Measurements using 633 nm laser with 1% power allowed by the ND filter; the laser is focused 30 μm above the surface.

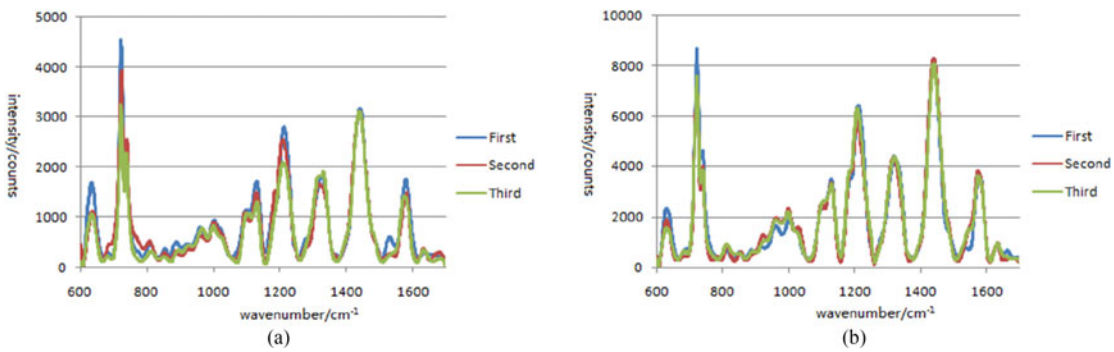

Fig. 5. Raman measurements of the sample using 785 nm laser. (a) uses ND filter that allows 10% laser power, focused on 20 μm above the sample surface. (b) uses ND filter that allows 20% laser power, focused on 30 μm above the sample surface.

Also, both Fig. 3(a) and (b) show relatively low signal intensity, especially on their first measurements (blue). They have Raman peaks that are about only 500 counts. Therefore, 532 nm laser might be unsuitable for stable Raman measurements of human blood serum.

When 633 nm laser is used, it is possible to avoid photo-degradation when laser is focused 30 μm above the substrate surface and the laser is at 1% power, as shown in Fig. 4. The three consecutive measurements produced spectra that are nearly identical.

When 785 nm laser is used, photo-degradation can be avoided when laser is focused 20 or 30 μm above the surface, using 10% and 25% laser power, respectively, as shown in Fig. 5. However, the

TABLE 1
Raman Peaks of Human Blood Serum Excited by 785 nm Laser

Position (cm^{-1})	Assignment
637	Uric acid
724	Hypoxanthine
812	Uric acid
853	Tyrosine/proline
886	Uric acid
957	Hypoxanthine
1004	Phenylalanine
1095	Hypoxanthine
1130	Uric acid
1206	Uric acid
1270	Amide III
1320	Hypoxanthine
1445	Hypoxanthine
1580	Hypoxanthine
1640,1677	Amide I

latter measurement condition shows both superior signal consistency and higher Raman peak intensity and is thus the better measurement condition when using 785 nm laser.

Table 1 shows a list of Raman peaks measured using 785 nm laser. Peaks are assigned according to recent studies; many Raman peaks are assigned to uric acid and hypoxanthine [5], [31], [32].

In conclusion, when the laser is focused at 30 μm above sample surface, both 633 nm at 1% power (power density is 26.3 W/cm^2) and 785 nm laser at 25% power (power density is 1420 W/cm^2) show promise in serum's Raman measurements; the shapes of their Raman spectra are also very similar. However, the latter setup produces much higher Raman intensity.

3.3 Sample Preparation: Serum Dropped on Substrates

As stated in the methods section, it takes four hours for the serum dropped on the substrate to dry; it is also easy to see visually that this method produces a thicker layer of diluted serum on the substrate.

The thick and uneven layer of serum on the substrate made SERS measurement ineffective, as shown in Fig. 6.

3.4 Serum Compositions

Because serum dropped on substrates were not able to produce satisfactory Raman measurement results, in this section, samples are prepared by immersing substrates in serum solutions.

Different serum compositions may severely affect the final enhanced Raman signal. When designing the experiment to find a good sample composition, many factors have to be taken into consideration. First, the SERS enhancement substrates used in this research are 5 $\times$ 5 mm in size, and the solutions must be 300 μl or more to have substrates fully immersed in the solutions; on the other hand, serum is scarce and must be conserved: only 2 ml of serum was obtained for each person from Zhejiang Cancer Hospital.

When colloidal substrate is used, Raman signal intensity is correlated with both the measured solution's concentration as well as the absolute amount of molecules that are measured. Thus, in this research, three sets of comparisons are made to investigate the following:

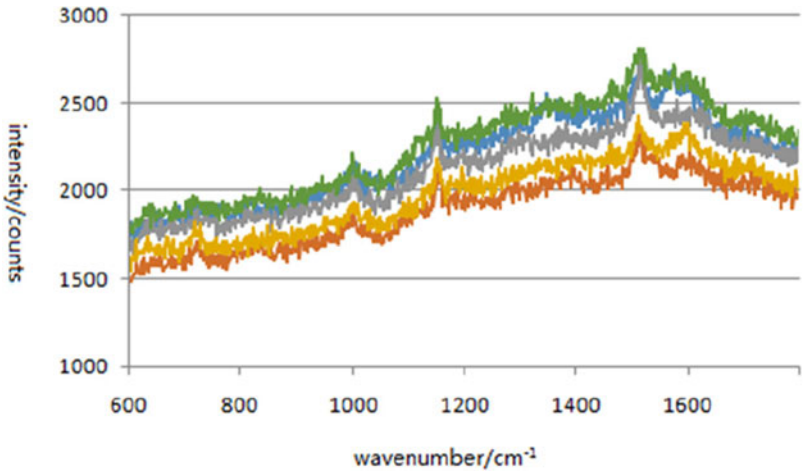

Fig. 6. Raman measurement of samples dropped on to the substrates.

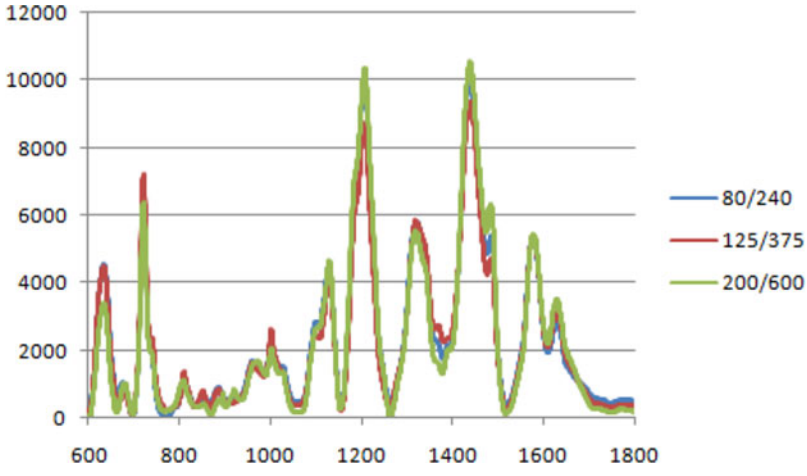

Fig. 7. Measurements with different serum volume and same serum concentration.

1) serum volume's effect on Raman intensity when serum concentration is constant;
2) serum concentration's effect on Raman intensity when serum volume is constant;
3) effect of different solvents.

Each set uses serum from one person to ensure individual differences between sera from different people does not interfere with measurement results. Set one is

1) 80 μl serum and 240 μl normal saline, concentration: 25%;
2) 125 μl serum and 375 μl normal saline, concentration: 25%;
3) 200 μl serum and 600 μl normal saline, concentration: 25%;

The Raman spectra of these three samples are compared to investigate how different volumes of serum would affect Raman signals, when serum concentration is at a constant – in all three samples, serum make up 25% of the whole sample. The result is shown in Fig. 7.

The comparison in Fig. 7 shows that when serum concentration is constant, serum volume does not have a significant effect on the signal intensity of the samples: the three spectra look almost identical.

Set two is

1) 125 μl serum and 125 μl normal saline, concentration: 50%;
2) 125 μl serum and 250 μl normal saline, concentration: 33%;

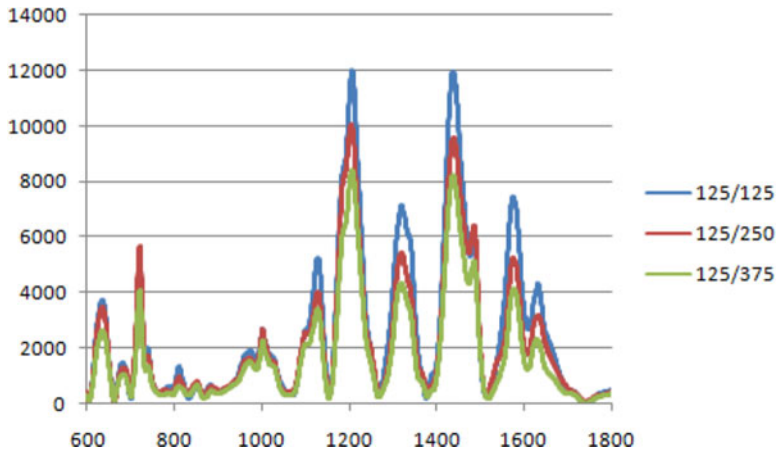

Fig. 8. Measurements with the same serum volume but different serum concentration.

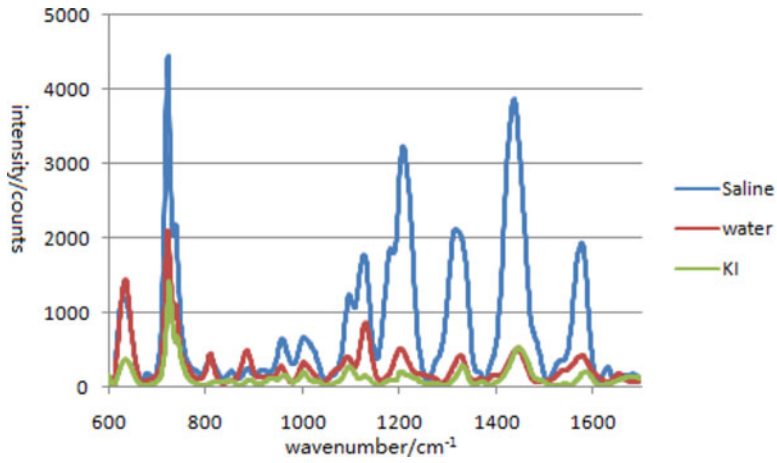

Fig. 9. Measurements using different solvents.

3) 125 μl serum and 375 μl normal saline, concentration: 25%;

The Raman spectra of these three samples are compared to investigate the effect of serum concentration on Raman signals when the amount of serum stays a constant. These three samples all use 125 μl of serum, but their serum concentrations are 50%, 33% and 25%, respectively. The result is shown in Fig. 8.

Result in Fig. 8 shows that when serum volume is constant, higher concentration contributes to higher Raman intensity. However, the relationship between concentration and Raman intensity might not be exactly linear; for example, the average intensity of the spectrum from sample with 50% concentration is 2612.7, and that from sample with 25% concentration is 1764.6. Thus, the average intensity of spectrum from 25% concentration sample is 67.5% of that from 50% concentration sample.

Set three is

1) 125 μl serum and 375 μl normal saline, serum concentration: 25%;
2) 125 μl serum and 375 μl deionized water, serum concentration: 25%;
3) 125 μl serum and 375 μl potassium iodide (KI) solution, serum concentration: 25%.

These three spectra are compared to investigate the effect of different solvents. Other than normal saline, deionized water is a very common solvent; also, different alkali halide solutions are used to enhance the Raman intensity of organic compounds on silver based substrates to different

degrees of success; therefore, another type of alkali halide solution, KI solution (1 mM KI, which is commonly used for enhancement) is used for comparison.

The result is shown in Fig. 9, which clearly shows that normal saline is the best solvent of the three. Measurement on sample that uses deionized water as the solvent has lower Raman intensity. On the other hand, sample that uses KI solution as the solvent produced spectra that suffer from much larger interference, which caused the Raman intensity to appear much smaller after baseline correction.

4. Conclusion and Discussions

In this research, two major problems in obtaining blood serum's Raman signal are investigated to find the optimal conditions for serum's Raman measurement; they are photo-degradation and serum sample compositions.

In order to avoid photo-degradation, 633 nm and 785 nm lasers are more preferable laser sources for serum measurements. For the 90 mW 785 nm laser, up to 25% laser power can be used in this setup, thus producing much higher Raman intensity than the 50 mW 633 nm laser, for which only 1% laser power could be used.

Thus, the recommended laser setup is 90 mW 785 nm laser with 25% ND filter, focused 30 μm above the substrate surface.

Several previous researches, such as Ito *et al.* study [33], may have suffered interference from photo-degradation; in this research, photo-degradation can be avoided, and serum's Raman peaks are found and shown in Table 1.

There are quite a few constraints in finding an optimal serum solution composition: serum is often scarce; on the other hand, the volume of the sample cannot be too low, or the substrate may not fully immerse in it.

Experiments show that lower serum concentration would reduce the signal intensity of Raman measurements. Thus, when the volume of serum is fixed, reducing the amount of solvent (in serum's case, normal saline) could increase Raman intensity, provided that there is enough solution in total to fully immerse the SERS substrate.

Currently, the serum solution composition suggested in this research is 125 μl serum and 250 μl normal saline. This composition has good serum concentration (33%) and can ensure that a 5 $\times$ 5 mm SERS substrate can fully immerse in the serum solution in a 1.5 mL micro centrifuge tube.

Using the currently recommended laser and solution composition setup as a standard, intense and clear serum spectra can be obtained. There are more identifiable serum Raman peaks in this study than in several previous studies, such as Harris *et al.* work [34].

Serum Raman peaks can also be identified consistently. There are a few different and hard to assign Raman peaks in 1300 to 1450 cm^{-1} range in Duo *et al.* different studies [35], [36]. In this research, 1320 and 1445 cm^{-1} peaks are found consistently and assigned confidently to Hypoxanthine.

Using the recommended setup, 216 different serum samples are measured. For each sample, five different spots on the sample are measured; averages and standard deviations of six typical serum's Raman peaks: 638, 725, 1005, 1130, 1205 and 1445 cm^{-1} are collected. From the six Raman peaks of the 216 samples, the maximum standard deviation is 21.8% and average standard deviation is 11.6%, which shows promising stability and repeatability.

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