Proceedings of The National Conference
On Undergraduate Research (NCUR) 2020
Montana State University, Bozeman MT
March 26-28, 2020

The Detection of Trace Levels of Epidermal Biomarkers Using Surface Enhanced Raman Spectroscopy (SERS) and its Applications in Early Skin Cancer Screening

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Abstract

In the present study, beta-carotene was analyzed by near infrared surface enhanced Raman Spectroscopy (SERS) to determine if trace amounts of the compound could be detected. Beta-carotene is a biomarker for certain types of epidermal cancer; therefore, the research was conducted in order to determine if Raman technology could provide a non-invasive method for detecting skin cancer. Throughout this process, all samples were analyzed by a handheld Raman spectrometer at 1064 nm. Beta-carotene was first analyzed as a solid to obtain a comparative Raman spectrum. The powder was then dissolved in chloroform to make reduced concentrations and better represent what is seen in skin cells. For 1000 ppm samples and all lower concentrations, the samples were surface enhanced by gold nanoparticles to gain clarity of the spectra. Signal averaging was performed to see the additive results of multiple Raman scans as well as increase the signal to noise ratio. With surface enhancement, the Raman spectrometer was able to analyze samples down to a concentration of 100 ppm. Further enhancement techniques are being studied to determine if lower concentrations will be detectable and other epidermal biomarkers are being researched for the same purpose.

Keywords: Epidermal, Biomarkers, Cancer

1. Introduction

Clinically proven as the most common form of malignancy, skin cancer in all of its forms accounts for 5.4 million cases and 10,000 deaths per year in the United States alone[1]. While the resulting death rate from these cases seems very small by comparison, the number of deaths could go down significantly if the methods for early detection and detection in general were improved.

There are three major types of skin cancer: Basal Cell Carcinomas (BCC), Squamous Cell Carcinomas (SCC), and Malignant Melanomas[2]. Each of these malignancies has a different pathway of growth and affects the human body in different ways. All three types can be detected and treated successfully; however, if left untreated, they can spread to other parts of the body and potentially become deadly very rapidly as they bore down in the body from the originating surface layer. The result of this is cancerous growth throughout the body that solely originated from a relatively small lesion on the skin. Some physical signs exist for each of these, including actinic keratosis and dysplasic nevi which are precancerous skin areas[2]. With or without a precancerous area occurring, the early detection of skin cancers is key and can be achieved in more efficient ways than in the past.

Current Detection methods of skin cancer involve a visual inspection of the skin followed by a biopsy and screening by histopathology[1]. These methods are very time consuming, taking days and sometimes up to a week to complete. With this time delay, the cancer could be progressing and treatment could become more difficult before results are even returned. It has also been shown that even with excision, the diagnostic accuracy can be very low. In one study, it was proven that the correct diagnosis of malignant melanoma cases was only 49-81% among dermatologists[1]. Due

to low accuracy, unnecessary medical costs, and patient discomfort/pain, it is ideal to search for a better method to diagnose multiple types of skin cancer.

Raman technology could provide new directions and promise for a non-invasive, fast, and accurate skin cancer detection system. Raman Theory is a chemical concept that describes the signal given off by excited molecules that are relaxing back to the ground state. The theory describes that electrons are excited up to a "virtual" energy level that does not reach the first excited level- it exists somewhere between the ground state and the first level. Once excited to this state, the electron relaxes back down while giving off the "Raman signal." This signal is described as Raman scattering, and can be recorded and converted into a spectrum. This spectrum can be interpreted like other spectra such as IR and NMR, and the patterns shown are informative about the nature of the material studied. The Raman system can also identify unknown compounds, which is useful for cancer studies. The Raman signal is obtained by shining a laser on the object which causes the electrons to move to the excited state- then the system records the movement of the electrons as they relax back down. In skin cancer applications, a laser can be shined on the skin which will excite biological molecules on the surface of the epidermis. The relaxation of these molecules will then give off a signature signal which is recorded and converted into a spectrum. If the spectrum matches the signature of a certain type of skin cancer that is known, there is an instantaneous confirmation of the presence and type of skin cancer.

In this study, the effectiveness of Raman technology as a skin cancer detection agent was studied. In order to do this, beta-carotene, a common component of skin, was observed in varying concentrations with a Raman Spectrometer. To make the study as realistic as possible, various amounts of beta-carotene that would be expected in different types of skin cancer were made in solution and scanned. The normal amount of beta-carotene in skin cancers is approximately 0.1 ppm, and the Raman detection system was able to clearly detect the beta-carotene down to 100 ppm. Further detection methods are being developed to increase the sensitivity of this instrument and to see if the desired amount can be detected. A separate project is being conducted in which a biophysical model of the skin is being built to see if certain types of skin cancers can be detected from a model that is very similar to skin.

2. Materials and Methods

The following chemicals were used in this experiment: Beta-Carotene (Sigma-Aldrich USA) and Chloroform (Spectrum).

Figure 1: The Handheld PGR 1064 system. The laser is placed on top of the desired object and shot by pressing the "trigger" located underneath on the handle of the device. The device immediately returns data by giving a Raman spectrum of the scanned object and an identification if available in the library.

All samples were analyzed by a PGR 1064 Handheld Raman Spectrometer "THOR" provided by Chemring Detection Systems (Charlotte, NC), shown in Figure 1. The PGR 1064 has a spectral range from 350-1850 cm^{-1} with a 10 cm^{-1} spectral resolution. The system employs a 1064 nm wavelength laser that contacts and analyzes samples in approximately 2 seconds. The system stores and displays obtained spectra that are linked to a corresponding PGR 1064 computer program. This program is able to display spectra and allows diverse manipulation such as signal averaging, background subtraction, pixel cleaning, and unit adjustment. Through this tandem program, obtained spectra can be analyzed on a larger scale and useful changes can be made to make sense of the data.

All samples were prepared by scooping a small amount of beta-carotene onto a 7.4 cm x 7.4 cm sheet of wax paper (exact amounts of dry material were irrelevant as long as enough material was present to be detected by the spectrometer). Liquid samples of varying concentrations were prepared by dissolving solid beta-carotene in a low spectral conflicting solvent, determined to be chloroform. These solutions were then dropped onto filter paper using micropipettes. The amount of sample varied for multiple runs to detect spectral differences; these amounts were either 1 microliter or 10 microliters to observe the difference. The liquid was allowed to dry prior to being analyzed. Liquid samples were also used with SERS strips (Metrohm Raman), small pieces of filter paper that were infused with gold nanoparticles. These strips were standardized in advance by a 10-um solution of BPE (1,2-Di(4-pyridyl)ethylene) in water.

For both solid and liquid samples, the handheld spectrometer was allowed to warm up and then it was placed at a 90-degree angle over the sample. The tip of the spectrometer was then brought to the sample surface and held as the laser was activated. The laser scanned for a 2 second period and then the instrument was lifted. After a processing time of approximately 10 seconds, the Raman spectrum was displayed on the screen of the spectrometer. Multiple scans of all materials were taken for accuracy and later for a signal averaging process. Some materials, such as beta-carotene, are air and/or light sensitive. These samples were analyzed through indirect contact with the sample. In order to analyze these, a black box contained the sample and the Raman laser was shot through a hole in the black box surface. No detectable difference was seen between the direct and indirect sampling.

To closely analyze the obtained spectra, the data was brought up in the PGR 1064 computer system. Here, the data was analyzed and manipulated in order to interpret the results. Multiple scans of the same material were signal averaged in order to increase resolution and decrease noise. These averaged spectra also provided a good representation of what the Raman spectra of each material was supposed to look like. For samples with large amounts of solvent, the spectra of the solvent could be subtracted out of the overall spectra. To do this, both the sample spectrum and a pure solvent spectrum were uploaded. Through manipulation, the computer system reduced the signal of the solvent within the sample spectrum, allowing more of the pure sample to be seen.

3. Results

After a series of scans were taken, they were uploaded into the PGR 1064 computer system for data manipulation. Some scans proved to be very clear and useful by themselves, however; others needed clarification to understand what was being observed. The PGR system allowed for these types of manipulations and the findings are given below.

Prior to applying the SERS substrate to samples, a Raman scan was taken of beta-carotene as a solid to see its definitive and clear Raman spectrum. Each of the samples had a very clear spectra with only one image due to a high concentration of the material in the laser's range. Even so, the intensity of the signal was not high with a single image. Therefore, samples were often signal averaged over many spectra to increase the signal and reduce background noise. Figure 2 below shows a single shot image of solid beta-carotene unenhanced by SERS. Without magnification, the three characteristic peaks of the material are very easily seen at 1020 cm^{-1}, 1160 cm^{-1}, and 1525 cm^{-1} even though they are not very pronounced. The highest amplitude of these peaks is at 1525 cm^{-1} with a height of 2500 which represents the stretching of C=C bonds. The peak at 1160cm^{-1} is representative of C-C vibrations in the polyene chain and the peak at 1020 cm^{-1} is representative of the rocking modes of CH_3 groups on the polyene chain[3].

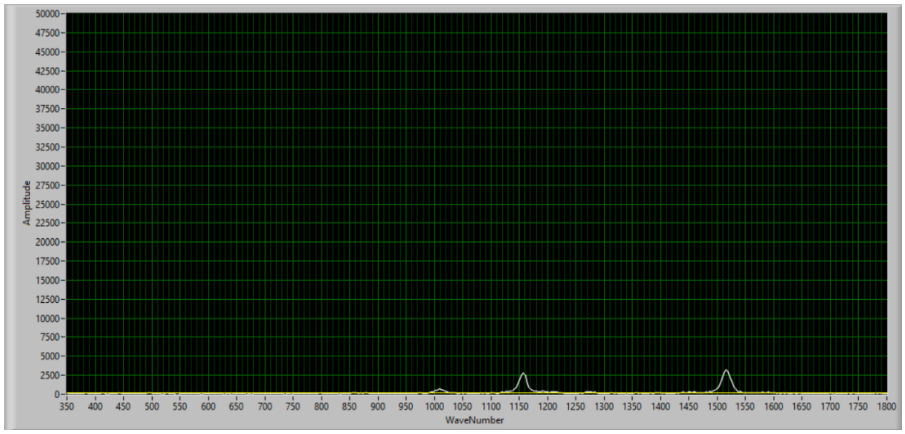

Figure 2: The Raman Spectrum of beta-carotene solid, unenhanced. Though the peaks are small, all three characteristic peaks of this material are able to be observed.

In order to obtain a more enhanced spectrum, many spectra were averaged together. The effect of this is that the important signals gain magnitude while the background noise is reduced. Signal averaging was performed on beta-carotene solid and the result is given in Figure 3 below. This image contains 30 scans of beta-carotene, with each successive scan added to the overall spectrum. While the spectrum shown in Figure 2 is sufficient to give the peaks of this material, more details including minor peaks are revealed with the use of signal averaging and this greatly aids material identification.

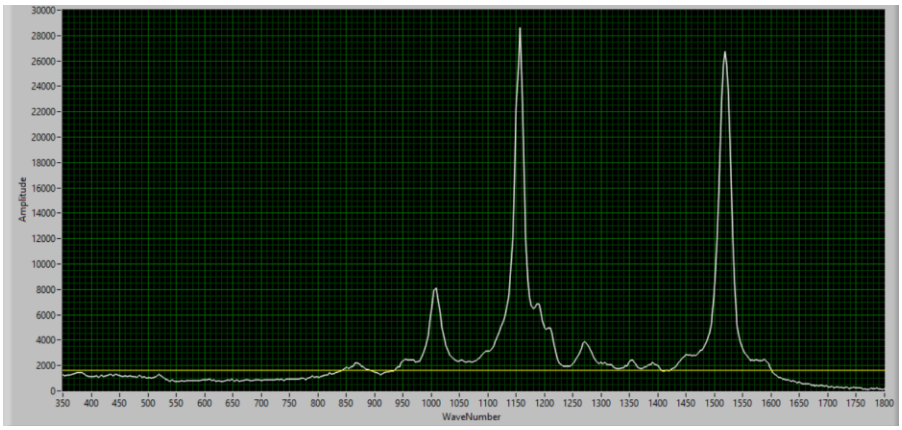

Figure 3. The Raman spectrum of beta-carotene solid, unenhanced, with 30 scans of material. The additive property of signal averaging allows for a much clearer image and brings out the minor peaks that begin to matter as well. The spectrum appears very clean and gives sufficient detail to be used as a comparison standard.

In order to enhance the single spectrum of beta-carotene in solution, a single file subtraction method was used. The PGR computer program has the ability to subtract signal from a spectrum by pulling out the peaks of an unwanted material (in this case, chloroform). Though this method only works for one spectrum at a time, the function is useful because the adjusted spectra can be averaged together for a cleaner final image. This also became especially useful when the concentrations of beta-carotene in solution became very low and the mole fraction of it was miniscule as compared to the chloroform it was dissolved in. Figures 4.1-4.3 give the individual spectra of pure chloroform and a sample of beta-carotene in solution at 1000 ppm. This is followed by the adjusted spectrum which had subtracted the chloroform peaks from the solution and left behind the desired beta-carotene. While this method is not perfect, there is a distinct difference between the non-subtracted spectrum and the adjusted one. Though only two of beta-carotene's characteristic peaks can be seen clearly in the final spectra, this method shows promise to work again with some improvements.

Figure 4: Spectrum of beta-carotene at 1000 ppm in chloroform. Two of the characteristic peaks can be easily observed at 1160 cm^{-1} and 1525 cm^{-1}.

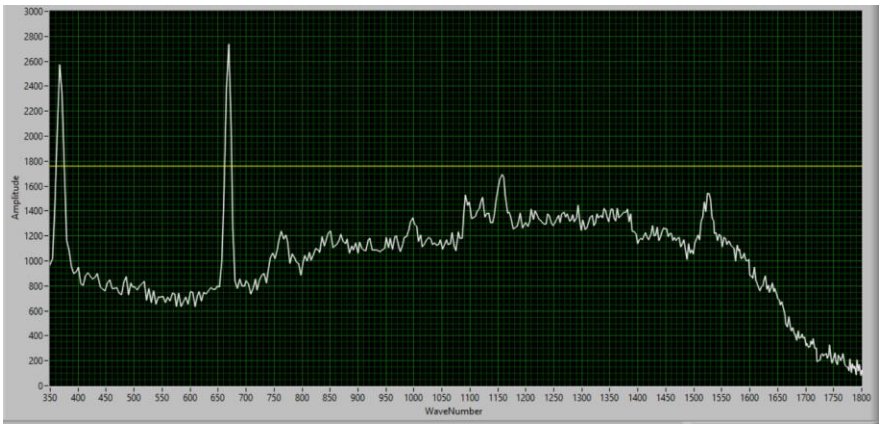

Figure 5: Spectrum of pure chloroform (Spectrum, USA) taken by dotting the liquid on wax paper.

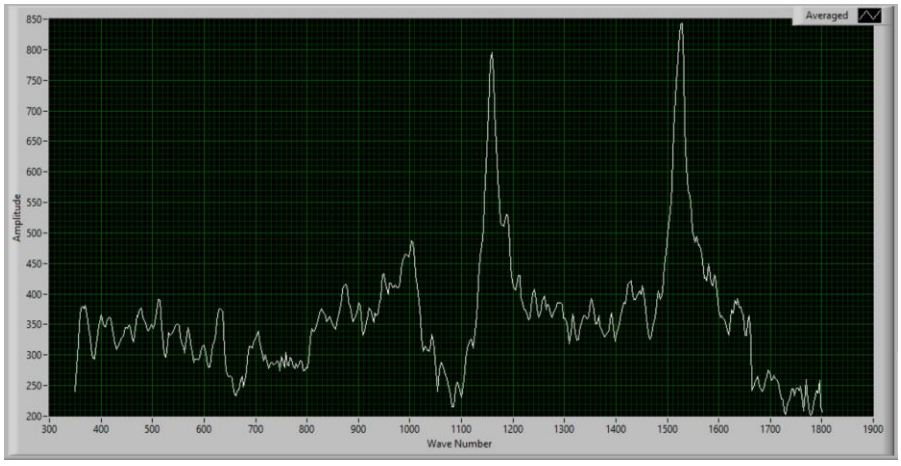

Figure 6: Spectrum of Beta-carotene with pure chloroform subtracted. The resulting image is not very clear and contains many unknown areas/peaks, but with more fine-tuning this method could show promise for cleaning up low mole fraction solutions like the one analyzed here.

After obtaining data for the beta-carotene without any enhancement, the SERS substrate was applied to identify the difference in signal between samples of lower concentrations. The SERS substrate is able to enhance the signal of molecules due to its ability to reduce vibrations and noise caused by conjugated bonds[4]. The substrate can achieve this by bonding to the desired molecule and locking it in a very specific conformation so that it cannot move[4]. The result of this reduction is larger signal peaks and less noise on the spectrum. Figures 5.1 and 5.2 give the results of scans of beta carotene at 2000 ppm unenhanced and 1000 ppm SERS enhanced, respectively.

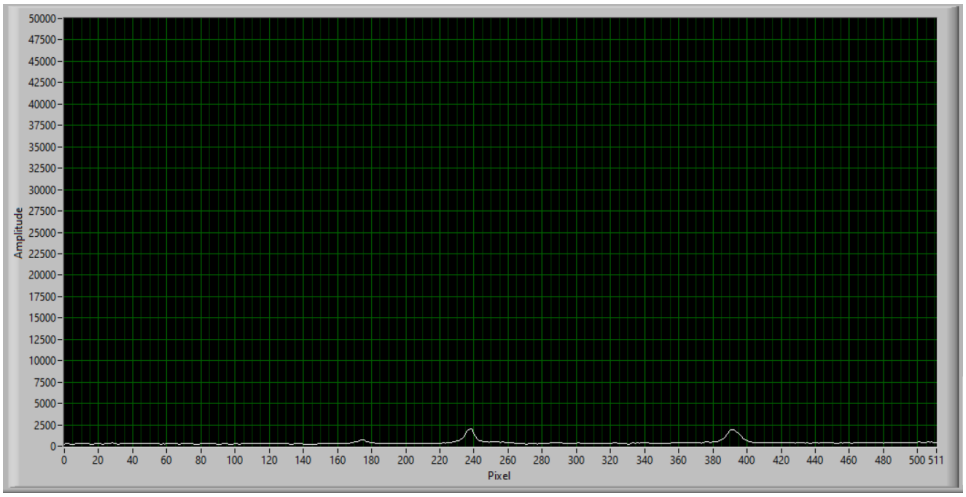

Figure 7: A single scan of beta-carotene at 2000 ppm, unenhanced. Though the major peaks are very small, their location is expected and they are visible.

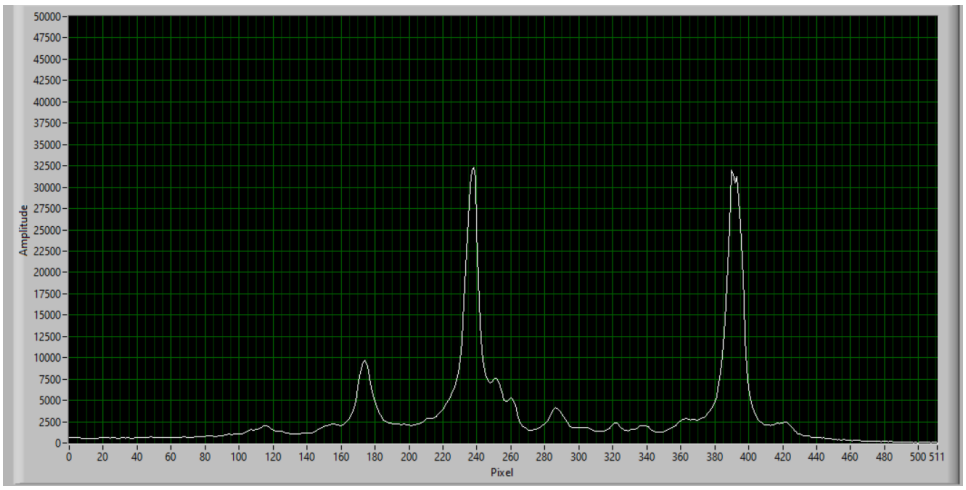

Figure 8: A single scan of beta-carotene at 1000 ppm, enhanced by the SERS substrate. At an even smaller concentration, it is very easy to see the defined peaks at a much larger amplitude. The SERS substrate locks the beta carotene into a certain conformation that allows for significantly lower vibrations and movement, resulting in a much larger spectrum.

From observing these spectra, it is very easy to see the effect of the SERS substrate on the beta-carotene samples. From the unenhanced sample to the enhanced sample, there is an amplitude gain of ~30,000. The largest peak on the unenhanced spectrum reaches ~2500, where the largest peak on the enhanced spectrum reaches ~32,500. This 13x magnification is very strong evidence that the SERS substrate is an effective method to enhance Raman spectra and effectively eliminate many factors that cause signal to be weak.

4. Discussion

Based on prior literature[4], the spectra found for beta-carotene were very accurate. All three defining peaks were present and located at the proper wavelengths in all of the observed samples. Given the resulting spectra displayed above, there is promise in the detection of small concentrations of beta-carotene and other small biological molecules utilizing a Raman method. While the detection limit for beta-carotene using Raman spectroscopy was only 2000 ppm, the SERS was able to detect down to 100 ppm, providing a massive improvement in the clarity and detection limit of the beta-carotene.

The importance of detecting biological molecules at low concentrations is that these exist at relatively low concentrations in normal skin; however, when skin is affected by a malignancy, these concentrations change. These changes are detectable and are what allow for the detection of skin cancers, but the physical change is almost miniscule. This is why it is important to have a detection system with both very good precision and accuracy in order to make a clear diagnosis. In this experiment, beta-carotene was able to be accurately detected down to 100 ppm. While this is good sensitivity for the instrument and its normal chemical uses, the detection limit must be reduced to 0.1 ppm in order to be helpful in differentiating skin lesions.

In order to plausibly use this technology, the next step to take would be to find a method to lower the detection limit of the Raman system. While this might only be achievable by a technology upgrade, this would be worthwhile so that a more efficient method of skin cancer detection could be made available. Even so, it was a surprise to achieve such a low beta-carotene detection limit with the current Raman spectrometer, excluding the accompanying SERS technology.

The expectations going into this project were high, and they were met. The handheld Raman technology used in this experiment had previously been used for other chemical applications, but not for the purpose of skin cancer screening. Given that, it was a surprise to see that beta-carotene was in the database of the system and the handheld could detect it in one scan. Moving forward, other skin cancer biomarkers could be detected through this method and could help bolster the results given. The end goal of this research is to synthesize the beta-carotene data with data to be collected from other biomarkers in order to program a Raman system for detection of various types of skin cancer. This type of detection system would be fast, effective, and painless for patients. This system could also greatly increase diagnostic accuracy and remove some uncertain characteristics of current detection methods. Overall, a SERS system would have many advantages as compared to current biological methods used to detect skin cancers.

5. Conclusion

This experiment provided significant evidence that SERS is an effective method to enhance Raman spectra and receive clearer results from single scans of material. The SERS substrate was very effective in stifling the vibrations and movement of molecules, providing a still scan and very amplified peaks as a result. This information is very helpful moving forward with the opportunity for this technology to be used as a method in early skin cancer detection. Because this method provides instantaneous results and can be used on the surface level, there is immense interest in the creation of a Raman Spectrometer that can identify skin cancers using this same technology.

6. Acknowledgements

This research opportunity was provided by the Wingate University Honors College. The funding for this research study was provided by the Wingate University Department of Chemistry and Physics. The authors of this project would like to thank Chemring Detections Systems for providing the handheld Raman Spectrometer and accompanying computer program for analysis. Morse would also like to thank Wingate University student Shelby Pasley for background, training, and prior research performed with the Raman technology.

7. References

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