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Abstract. We have recently applied the technique of drop coating deposition Raman (DCDR) spectroscopy for colorectal cancer (CRC) detection using blood plasma. The aim of this study was to develop a more convenient and stable method based on blood plasma for noninvasive CRC detection. Significant differences are observed in DCDR spectra between healthy ($n = 105$) and cancer ($n = 75$) plasma from 15 CRC patients and 21 volunteers, particularly in the spectra that are related to proteins, nucleic acids, and β-carotene. The multivariate analysis principal components analysis and the linear discriminate analysis, together with leave-one-out, cross validation were used on DCDR spectra and yielded a sensitivity of 100% (75/75) and specificity of 98.1% (103/105) for detection of CRC. This study demonstrates that DCDR spectroscopy of blood plasma associated with multivariate statistical algorithms has the potential for the noninvasive detection of CRC. [DOI: 10.1117/1.JBO.20.3.037004]

Keywords: drop coating deposition Raman spectroscopy; colorectal cancer; blood plasma; principal components analysis; linear discriminate analysis.

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1 Introduction

As an optical spectroscopic and/or imaging technique, Raman spectroscopy, which can probe molecular vibrations of biological samples that provide a highly specific fingerprint of the molecular structure and biochemical composition without external markers, has been accepted to be a powerful tool for bioanalytical and biomedical applications. Near-infrared (NIR) Raman spectroscopy has certain advantages over Fourier transform IR spectroscopy in tissue diagnosis because of its relative insensitivity to water and deeper penetration in the tissue using NIR excitation. As such, for a long period of time, NIR Raman spectroscopy has attracted much attention for *in vivo* and *in vitro* diagnosis in a variety of cancers including lung,[1] cervix,[2] skin,[3] nasopharynx,[4] and gastric[5] cancers. As for colorectal cancer (CRC), the third most common cancer worldwide, several Raman studies of human CRC tissue and cells were explored.[6–9]

Besides tissues and cells, Raman spectroscopy of biofluids such as tears,[10] urine,[11] bile,[12] and serum,[13,14] have also been investigated. Among these, serum/plasma attracted the most attention, because it is noninvasive and easily accessible and is expected to contain markers shed from tumor cells into the circulation.[15] However, NIR Raman signals are inherently weak, and additionally, blood serum/plasma suffers strong fluorescence background along with the Raman spectra, all of which make it difficult to extract the Raman signals. With the progress of surface enhanced Raman spectroscopy (SERS) technology, the low intensity of Raman scattering and the strong autofluorescence has been resolved. Some remarkable achievements of SERS to serum/plasma have been reported.[16–18] It is worth mentioning that gold nanoparticle based SERS of serum combined with principal component analysis (PCA) and linear discriminant analysis (LDA) yielded a diagnostic sensitivity of 97.4% and specificity of 100% for separating CRC samples from normal samples.[19]

However, the key limitations of SERS include the generation of spurious background signals, optical damage susceptibility, and often poor reproducibility in terms of both absolute intensity and spectral shape.[20] A newly developed method called drop coating deposition Raman (DCDR) could increase the intensity of Raman scattering of analytes by preconcentration and would be a proper technique for medical diagnostic tests using body fluids. DCDR is a method in which a microvolume of solution is deposited on a suitable substrate, followed by solvent evaporation and nondestructive Raman detection.[20] After this method has been presented, its application on protein mixtures,[21,22] tears,[23] and serum[24] has been reported. The results of these studies suggest that DCDR spectroscopy could be an effective technique capable of assisting the diagnosis and screening of cancer.

In this study, the DCDR method is used on plasma for the identification of CRC. Multivariate statistical techniques, including PCA and LDA, were employed to develop effective diagnostic algorithms for differentiations among two groups.

2 Materials and Methods

2.1 *Raman Spectroscopy*

Raman spectra were recorded with a confocal Raman microspectrometer (Renishaw, InVia+Plus) that mainly consists of a 785-nm diode laser, a transmissive imaging spectrograph with an NIR-optimized, back-illuminated, deep-depletion

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charge-coupled device (CCD) detector, and a single 1200 lines/mm dispersion grating. A 50× objective was used to collect the back-scattered radiation. The spectra were acquired in static mode, with a center at 1200 cm^{-1} which covered the range 608 cm^{-1}- to 1720 cm^{-1}, and the spectra resolution is 1 cm^{-1}. In order to obtain a better signal-to-noise ratio, 40 mW laser power on the sample and a 2×10 s acquisition time were used to produce each spectrum. Before Raman scanning, the instrument was calibrated by silicon at 520 cm^{-1}.

2.2 *Subject and Protocol*

In this study, two groups were included: 15 patients (mean age 54 years) with confirmed clinical and histopathological diagnosis of CRC (TNM-based stage groupings: stage III or stage IV) and 21 healthy volunteers (mean age 38 years) as the control group. All patients were from the Zhujiang Hospital, Guangzhou, China. All of those patients have signed an informed consent form to permit the investigative utilization of their blood.

After overnight fasting, single 5 ml peripheral blood samples were obtained from the patients and the volunteers between 7:00 to 9:00 AM with the use of ethylenediaminetetraacetic acid as an anticoagulant. Blood cells were removed by centrifugation at 3000 rpm for 20 min to obtain the blood plasma. Aliquots of the samples were frozen at −80°C before Raman spectroscopy analysis was performed.

2.3 *Sample Preparation*

Frozen plasma samples were thawed and 10 μl of each sample was deposited on an aluminum substrate using a calibrated micropipette. The aluminum substrates were cut into a size of 5×5 mm^2 to gather the deposited plasma in a smaller area and were placed over the glass slide before deposition. The samples were dried in air at temperature- and humidity-controlled laboratory conditions for 1 h without light and considerable vibration. Then five-point Raman spectra were randomly recorded from the periphery of the dried sample surface.

2.4 *Data Preprocessing*

First, a specially designed software package (Raman Wire 3.2) is used to preprocess these original spectra, including the elimination of cosmic radiation, the subtraction of the CCD dark-noise, and the smoothing of the data. Second, the Raman spectrum of each sample is baseline corrected by the software R 2.8.1. Finally, the spectrum is normalized with respect to the total area under the Raman curve from 600 to 1700 cm^{-1}. Thus, the systematic deviation can be minimized and the relative Raman peak intensities among different samples can be achieved.

2.5 *Statistical Analysis*

In our study, spectral analysis was performed using the PCA and LDA techniques. First, since the large number of variables in our Raman data (1015 wavelengths per spectrum) and PCA is utilized to reduce the dimensionality of the dataset while retaining the most important information for characterization, PCA can generate a series of PCs which are linear combinations of initial variables with the first PC explaining the highest variance of the data, and the others decreasing. Then the most discriminant PCs for differentiating spectra from both groups of CRC and normal volunteers were identified using unpaired Student's *t*-test ($p < 0.05$). Second, these significant PCs scores were loaded into the model of LDA, which is a supervised analytical technique that could be used to develop diagnostic algorithms using these PCs scores. LDA determines the discriminant function that maximizes the variances in the data among groups while minimizing the variances between members of the same group. The performance of the PCA-LDA diagnostic algorithm was validated using the leave-one subject-out, cross-validation methodology. In this method, five spectra from the same subject were removed out of the data set, and the PCA-LDA algorithm was developed using the remaining spectra ("training set"). Then the five spectra were input to the PCA-LDA algorithm and a classification output was obtained. This process was repeated until all withheld spectra were classified. Finally, to compare the performance of the PCA-LDA model for blood plasma classification, the receiver operating characteristic (ROC) curves were generated by successively changing the thresholds to determine correct and incorrect classifications for all subjects. Statistical Package for the Social Science (SPSS) and MatLab software were used for statistical analysis in this study.

3 Results and Discussions

3.1 *Spectral Features*

Figure 1 shows the average standardized Raman spectra obtained from the 21 normal subject blood plasma samples and the 15 colonic cancer patient plasma samples. Prominent Raman peaks can be observed in plasma samples at the following locations with their respective tentative biochemical assignments:[5,19,25] 881 cm^{-1} [tryptophan(Trp)], 936 cm^{-1} (C—C stretching in α-helix conformation of proteins), 1004 cm^{-1} [C—C symmetric stretch ring breathing of phenylalanine (Phe)], 1155 cm^{-1} (β-carotene, C—C skeletal stretch), 1208 cm^{-1} (C—C_6H_5 stretching mode of Phe and Trp), 1323 cm^{-1} (CH_3CH_2 wagging and deforming in collagen and purine bases of nucleic acids), 1335 cm^{-1} (CH_3CH_2 twisting mode of proteins and nucleic acids), 1445 cm^{-1} (CH_2 bendingmode of proteins and lipids), 1523 cm^{-1} (β-carotene) 1551 cm^{-1} (Trp) and 1660 cm^{-1} (amide I band C ¼O stretching mode of proteins, mainly indicated in α-helix conformation). Despite the similarity in shape and intensity of the characteristic spectra of normal and cancer plasma, remarkable changes of Raman peak intensity and bandwidths are observed. First, compared with the healthy group, the cancer group shows higher intensities at proteins [amide I (~1660 cm^{-1}), CH_2 deformation (~1445 cm^{-1}) and

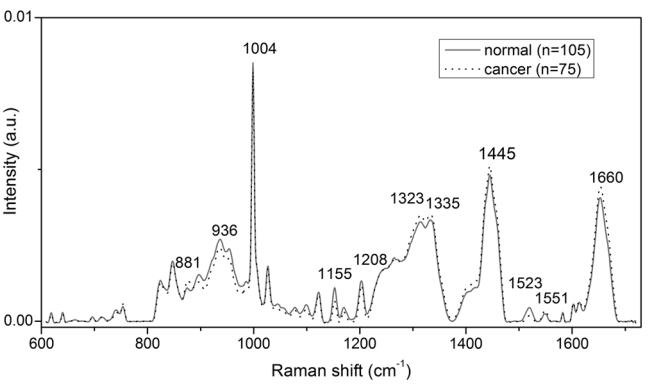

Fig. 1 Average standardized Raman spectra of normal subject blood plasma and colonic cancer patient plasma.

α-helix conformation (~936 cm^{-1})], DNA (~1335 cm^{-1}, and ~1323 cm^{-1}), and Trp (~881 cm^{-1}, and ~1551 cm^{-1}), while the intensities were lower at β-carotene (~1155 cm^{-1}, and ~1523 cm^{-1}), and Phe (~1004 cm^{-1}, and ~1208 cm^{-1}). These 11 significant Raman peaks in Raman spectra were also identified with statistically significant differences among the two groups using unpaired Student's t-test ($p < 0.05$). Second, slight changes of bandwidths were observed in the range of 920 to 980 cm^{-1} and 1640 to 1680 cm^{-1}.

The increased intensities of the 1335 cm^{-1} and 1323 cm^{-1} peaks are highly associated with a higher content of cell-free DNA. Although the reason is not known for sure, several cancer patients' blood have been proven to have an increased level of cell-free nucleic acids.[26] Many studies have shown strong associations between a higher level of β-carotene and a reduced incidence of many forms of cancer.[26,27] Serum Raman spectroscopy of CRC patients and healthy volunteer controls has been reported, and a lower level of β-carotene in the CRC group has been observed.[27] Most of these studies were excited by a visible laser, in which β-carotene shows a much stronger intensity because of resonance. In our study, near-infrared Raman spectroscopy has been used to generate Raman spectra, and the different spectra intensities of β-carotene in the cancer group and the healthy group are in concordance with existing reports.[27,28] The increased intensity and the bandwidth broadening of 1660 cm^{-1} indicate a higher level of α-helix proteins (e.g., histone, the main protein component that makes up the chromatin).[5] The strongest intensity at 1445 cm^{-1} in the Raman spectrum due to the CH_2 deformation was found to be higher in the cancer group, which is in agreement with a previous study of blood plasma with gastric cancer.[14] The higher intensity of tryptophan at 881 and 1551 cm^{-1} in the serum of CRC patients has also been reported.[25,28] Additionally, a reduced percentage of Phenylalanine (Phe, 1004 and 1208 cm^{-1}) in cancer conditions has been obtained in our study. Overall, for cancer conditions, increased levels of cell-free DNA and some proteins and a decreased level of β-carotene were observed in our study, which is in concordance with the existing reports.

3.2 *Multivariate Analysis*

We employ the multivariate statistical technique (i.e., PCA and LDA) by incorporating the entire Raman spectrum to determine the most diagnostically significant Raman features for plasma analysis and classification. Unpaired Student's t-test was used on the obtained PCs, and for the most of the training sets, six PCs (PC1, PC2, PC4, PC5, PC6, PC7) with a typical value of ~83.26% of the total variance were consistently selected as diagnostically significant ($p < 0.05$) for discriminating CRC plasma and normal plasma. The consistency of the PC sets suggests that the diagnostic algorithms developed were robust. Figure 2 shows the six significant PC loadings computed by PCA, in which PC1 accounts for the largest Raman spectral variance (~54.31% of the total variance), while the successive PCs describe the spectral features that contribute progressively smaller variances. The peaks and troughs in Fig. 2 indicate the positive and negative correlation between PCs and Raman bands. Using PC1 as an example, contributions from 881, 1004, 1323, 1335, 1445, 1551, 1660 cm^{-1} are mostly positively correlated with PC1, while those from 936, 1155, 1208, 1523 cm^{-1} are negatively correlated with PC1. PC loadings essentially reveal the significant Raman spectral features between those two different types of blood plasma and their

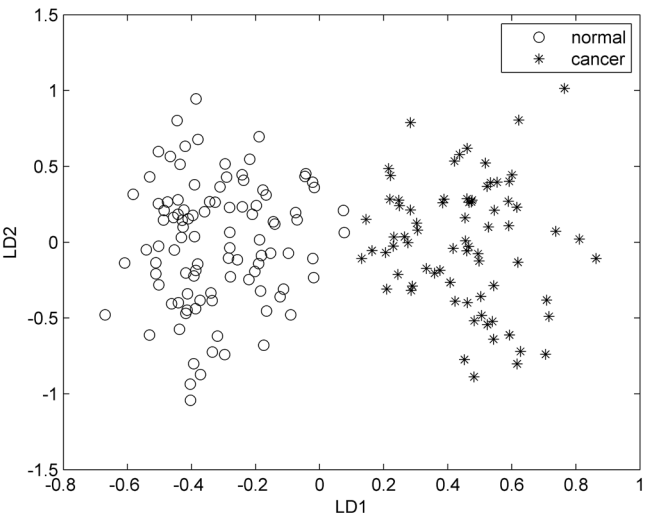

Fig. 3 Two-dimensional scatter plots of the two linear discriminant functions (LD1 versus LD2) belonging to the normal and colonic cancer using the PCA-LDA technique.

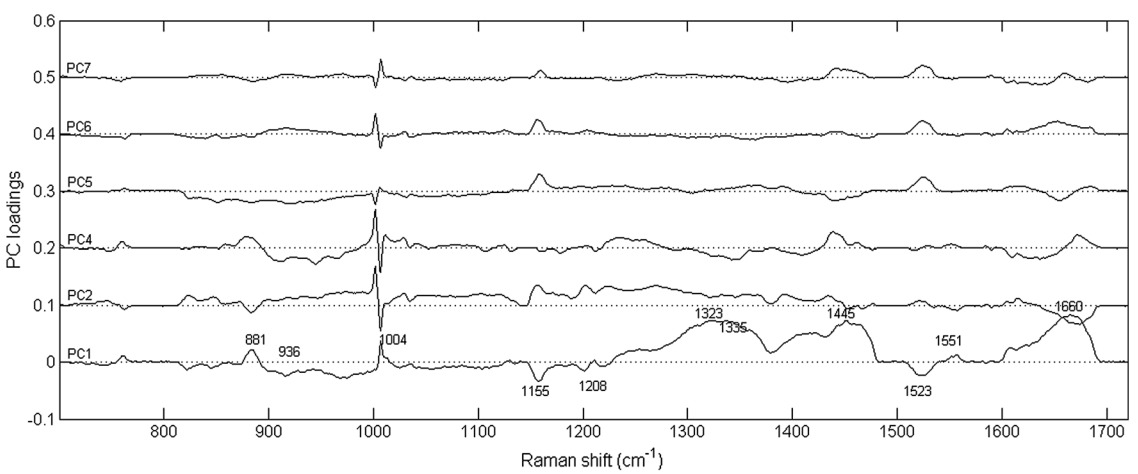

Fig. 2 The six diagnostically significant PC loadings (PC1, PC2, PC4, PC5, PC6, PC7) accounting for ~83.26% of the total variance calculated from blood plasma Raman spectra.

features are similar to those of blood plasma Raman spectra (Fig. 1), which indicate that the PCA model has the ability to extract the diagnostically significant information from the data set. These six significant PCs were loaded into the LDA model for generating effective diagnostic algorithms for plasma classification. Figure 3 exhibits the two-dimensional scatter plot of the two linear discriminant (LD) functions (LD1 versus LD2) based on the PCA-LDA model, which shows that healthy and cancerous plasma can be largely clustered into two separate groups. The PCA-LDA algorithm together with leave-one-out, cross-validation method based on DCDR spectroscopy provides a diagnostic sensitivity of 100% (75/75) and specificity of 98.1% (103/105) for distinguishing healthy and CRC plasma. To further evaluate the performance of the PCA-LDA based diagnostic algorithm for CRC diagnosis, the ROC curve was generated from the scatter plot in Fig. 3 at different threshold levels and the area under the ROC curve is 0.999. The result shows that the blood plasma spectra can be used for CRC detection with high sensitivity and specificity.

4 Conclusion

In summary, our study shows that blood plasma DCDR spectroscopy together with PCA-LDA algorithms may be a suitable approach for noninvasive and convenient detection of CRC. According to our study, an increased level of cell-free DNA and a decreased level of β-carotene were observed. The integration area under the ROC curve of 0.999 further demonstrates tremendous promise for the development of blood plasma DCDR analysis into a clinical tool for cancer detection and also for the screening of several types of cancer. More data is necessary for blood plasma Raman spectra as a tool for cancer detection in the future.

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