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Review article
Pregledni članak

RAMAN SPECTROSCOPY AS A NEW BIOCHEMICAL DIAGNOSTIC TOOL

RAMANSKA SPEKTROSKOPIJA KAO NOVO BIOHEMIJSKO DIJAGNOSTIČKO SREDSTVO

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Summary

In this review, Raman spectroscopy is described as a new and potentially powerful diagnostic tool in comparison to routine biochemical tests. Advanced instrumentation and new Raman spectroscopy techniques enable rapid and simultaneous identification and/or determination of several biochemical parameters, such as glucose, acetone, creatinine, urea, lipid profile, uric acid, total protein, etc, with a very low limit of detection. Raman spectroscopy could also be applied in molecule and cell characterization, as well as diagnostics of atherosclerosis in its early stage. Raman spectroscopy is non-destructive and could be applied to all kinds of samples, which simplifies the diagnostics of numerous diseases and pathologic states. Special attention is paid to literature data illustrating the application of Raman spectroscopy for transdermal glucose monitoring and cancer diagnostics.

Keywords: biochemical parameters, cancer diagnosis, glucose monitoring, Raman spectroscopy

Kratak sadržaj

U ovom prikazu opisana je primena Ramanske spektroskopije kao nove metode velikih mogućnosti u dijagnostici, u poređenju sa rutinskim biohemijskim testovima. Metoda je razvijena i usavršena za identifikaciju i/ili određivanje velikog broja biohemijskih parametara, kao što su glukoza, aceton, kreatinin, urea, lipidni profil, mokraćna kiselina, ukupni proteini i drugi, uz veoma nizak limit detekcije. Ramanska spektroskopija takođe se može primenjivati u molekulskoj i ćelijskoj karakterizaciji, kao i za dijagnostiku ranog stadijuma ateroskleroze. Ramanska spektroskopija je nedestruktivna i može se primenjivati na sve vrste uzoraka, što pojednostavljuje dijagnostiku brojnih bolesti i patoloških stanja. Posebna pažnja u radu je posvećena podacima iz literature koji ilustruju primenu Ramanske spektroskopije u transdermalnom monitoringu glukoze i dijagnostici kancera.

Ključne reči: biohemijski parametri, dijagnostika kancera, monitoring glukoze, Ramanska spektroskopija

Introduction

An ideal method for the identification and determination of important biochemical parameters should be fast, reliable, specific, accurate and as low-cost as possible. The advantage of some spectroscopic methods is in avoiding sample preparation and reagents for each parameter separately, in comparison to most routine tests based on chemical reactions. The fact that Raman spectroscopy is able to analyze samples without prior preparation in most cases makes this method suitable for biochemical diagnostics, and that is confirmed in this review by numerous literature data.

Based on the Raman effect (see *Figure 1)* new spectroscopic instrumentation was developed, and very soon became applicable, thanks to lasers developed as monochromatic light sources as well as more sophisticated detectors. The method is based on the intensity measurement of inelastic incoherent light after the sample is illuminated with a monochromatic light source. Obtained spectra enable qualitative and quantitative analyses.

The Raman technique was applied for biological molecules characterization very soon after the Raman phenomenon was discovered in 1928 (1). But for

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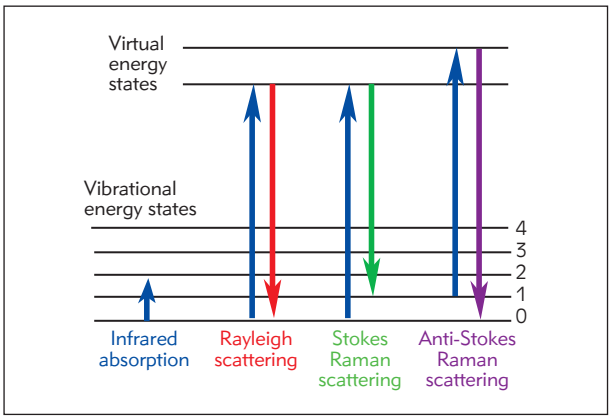

Figure 1 The Raman effect

decades, due to problems with spectra quality and/or their understanding, there was no practical application of Raman spectroscopy in biochemical diagnostics. A connection of physical and analytical chemistry with biochemical diagnosis is necessary, especially with a view to establishing practical applications of more sophisticated, reliable and low-cost instrumental techniques.

Raman spectroscopy has been used before for qualitative analysis. The advantages of newly designed instruments equipped with holographic optical elements, charge-coupled device cameras, long wavelength solid state lasers and chemometric methods, facilitate the measurement of concentrations with Raman spectrometers with a low limit of detection (LOD) in comparison to the other spectroscopic techniques (2). For more than a decade, Raman spectroscopy has been considered as a powerful diagnostic tool in biochemistry (3–7).

Nowadays, there are several advanced Raman spectroscopy techniques:

a) *Fourier Transform Raman Spectroscopy,* since the early 1980s;

b) *Near-Infrared Raman Spectroscopy* provides a good quality spectrum recorded from *in vivo* human skin in less than 1 s, enabling Raman data collection in the clinical surroundings with good S/N values at the same time;

c) *Resonant Raman Spectroscopy* allows determination of analytes with 10^2–10^6 times higher sensitivity than conventional Raman techniques, while the obtained spectra are simpler shaped than usual;

d) *Micro-Raman spectroscopy* can be applied for more specified investigation of biosamples, and leads to a completely nondestructive technique when combining laser tweezers with confocal micro-Raman spectroscopy;

e) *Resonant Micro-Raman Spectroscopy* achieves lowering of the sample mass required;

f) *Surface-Enhanced Raman Spectroscopy* (*SERS*) is a technique based on the phenomenon that a compound adsorbed to a metal surface has 10^3–10^6 times stronger Raman scaterring, so the obtained spectra may give much more information.

Literature data describe the potential application of Raman spectroscopy in the diagnosis of atherosclerosis, analysis of biological fluids (with special attention on non-invasive glucose monitoring), microbial identification, diagnosis of skin diseases, and diagnosis of cancer. We review some of these here.

Biochemical Parameters

Raman spectroscopy allows very fast determinations in different biological samples, such as urine or whole blood. A study by Dou et al. (8) reports the possibility for quantification of components of human urine based on anti-Stokes Raman spectra, to determine the concentrations of glucose, acetone, and urea.

Raman spectroscopy is obviously a very powerful tool in biochemistry, especially if measured diagnostic parameters can be connected to prognosis of future state, particularly significant for arthrosclerotic conditions. For example, the precise definition and understanding of the lipid profile in high-risk groups of patients is very important. Raman spectroscopy studies were usually undertaken to clarify the structure of lipoprotein, and they were performed on bulk solutions of lipoproteins (9), giving as a result averaged information about the size and density of lipoproteins (10).

The most advanced applications of Raman spectroscopy nowadays are directed to atherosclerotic vascular disease. Current atherosclerosis research is focused on unstable plaques; a thin fibrous top over collected necrotic lipid material that mainly consists of cholesterol (11). Recent studies have shown that chemical composition and morphology determine atherosclerotic plaque instability, and predict disease progression and the risk of complications such as thrombosis and acute plaque hemorrhage. The progression and regression of atherosclerotic plaques appear to be related to the amount and type of lipids that accumulate in blood vessels (12).

Raman microspectroscopy has been used for *in situ* characterization of cholesterol crystals in artery endothelial cells (13). The study of Chan et al. (14) goes further showing that the biomolecular Raman spectroscopic fingerprint of individual very low density lipoproteins (VLDL) is unique, highly reproducible and can be used to monitor biochemical changes of the particles due to lipoprotein metabolism. Additionally, the free unsaturated and saturated fatty acids can pack into different phases, leading to the formation of a highly ordered saturated core, which can be detected spectroscopically. Any information in this

area can help in the creation of measurements for the monitoring of at-risk cardiovascular patients.

Raman spectroscopy is even able to give the answer to how advanced is the calcification process of atherosclerotic plaques in human coronary arteries (15, 16).

Glucose level data, as well as some other biochemical parameters, can be followed by the proposed technique, such as creatinine, urea and cholesterol, in addition to several other tissue diagnosis applications. For that purpose the application of a non-imaging optical element – compound hyperbolic concentrator (CHC) is proposed, which enables accommodation of a wide angular range of scattered photons from the biological tissue by conversion into a limited range of angles (17).

Near-infrared Raman spectroscopy can be a new technique for physical evaluations, allowing the measurement of lactic acid concentrations, in blood or muscles, during physical activity in a transcutaneous noninvasive way. Raman spectroscopy has been used to follow the content of lactic acid from an athlete without interrupting his exercise for sample collection (18). Experiments were undertaken to verify the presence of lactic acid in the Raman spectra of solutions of lactic acid in human serum and in blood from a Wistar rat. After these two experiments, another was developed *in vivo* in a Wistar rat, by injecting intraperitoneally 1 mL of a 0.12 mol/L lactic acid aqueous solution. An optical fiber catheter touching the skin of the rat groin over the ileac vein collected the Raman signal.

Glucose Monitoring

The monitoring of glucose concentrations in patients who suffer from diabetes has been established as imperative, as a result of some early studies showing that tight control of blood glucose concentrations, by frequent testing of the glucose level and adequate corrections of insulin doses, decreases the possible long-term complications diabetes could cause (19).

A study performed by Rohleder et al. (20) concerned Raman spectroscopy as a reagent-free tool for predicting the concentrations of several biochemical parameters in serum and/or serum ultrafiltrate. For samples from 247 blood donors, the concentrations of glucose, triglycerides, urea, total protein, cholesterol, high density lipoprotein, low density lipoprotein and uric acid were determined with accuracy within the clinically interesting range. Relative errors of prediction, based solely on the Raman spectra, were around 12%. This study also showed that ultrafiltration can efficiently reduce fluorescent light background to improve prediction accuracy. Raman spectroscopy was presented as a powerful diagnostic technique when costs and time of analysis take precedence over high accuracy.

Classic invasive blood sample collection is painful, discomforting, could be connected with infections, requires sharp objects, and may result in a patient's avoiding of frequent glucose level checking. Finding some noninvasive procedure for measuring glucose would be a great benefit for the millions of people affected by diabetes.

Several techniques seemed to be useful for the abovementioned purpose, such as infrared spectroscopy, fluorescence spectroscopy, electrochemical measurements, NMR spectroscopy and some others, but for the time being all of them are not convenient for regular application.

Some implantable sensors for glucose may be efficient, but they are certanly invasive (21). The level of glucose in blood corresponds to the glucose level in interstitial fluid, and makes a base for the transcutaneos determination of glucose by Raman spectroscopy. Calculations confirmed that about 30% of Raman spectra intensity collected *in vitro* could be collected in interstitial fluid *in vivo* (22).

In order to obtain good quality – »useful« Raman spectra, it is necessary to use high excitation power and long time of signal collection. The imperative is to stay within a safe level of irradiance and be sure that no skin irritation or damage could occur. According to the American National Standards Institute, skin exposure to an 830 nm laser beam for 10 s at 0.36 W/cm^2 is marked as level of comfort (23).

A Raman signal obtained by measuring biological samples, such as skin, is very weak and overlapped by strong fluorescence signals. Fluorescence background is partly reduced by using an excitation laser at 830 nm. Further, fluorescence background varies from sample to sample, and with the exposure time, as well. This is the reason why it is necessary to remove the influence of broad fluorescence background by a high-pass filter. As an example procedure, after least square fitting a fifth order polynomial spectral curve, and subtraction of raw skin spectrum, only a sharp Raman spectrum remains (24).

Raman bands are specific to glucose molecular structure. The vibrations monitored in Raman spectra are fundamental and thus are sharper. Further, water has a low Raman cross-section as opposed to its high IR absorption. It is possible to detect glucose by monitoring the 2900 cm^{-1} COH stretch band or the COO and COC stretch Raman bands at 900–1200 cm^{-1}, which represents a fingerprint for glucose (25).

At the present time, a non-imaging optics based portable Raman spectroscopy instrument has been constructed with the aim of measuring transdermal blood glucose (17). The proposed non-imaging optical element is a so-called compound hyperbolic concentrator, and at the same time is able to follow some other biochemical parameters (creatinine, urea and cholesterol).

Although the proposed method seems promising, it is still the subject of testing performed by tissue phantom, animal model, and human subject studies (26).

Raman Spectroscopy in Cancer Diagnosis

Innovations in the Raman spectroscopic instrumentation have improved the sensitivity of the measurement and allowed the obtaining of useful spectra from biological tissue and cells. Several studies have confirmed the possible use of Raman spectroscopy for the identification and classification of malignant changes (27–29).

All cancer stages are followed by fundamental changes in cellular morphology and/or tissue biochemistry. During the process of carcinogenesis, some changes in the distribution of DNA, lipids and proteins in the cells occur. These could be early markers in the detection of high-risk patients before morphological changes appear. A study by Shetty et al. (30) is an important contribution to establishing these biochemical changes. Further understanding of the carcinogenesis process would help to improve the diagnostic techniques, thus improving survival.

Beside diagnostics, Raman spectroscopy can be applied in cancer biology in the detection of specific changes in the structure of DNA or proteins, and thus may be used to follow the chemotherapeutic action of several drugs (31).

Studies in which Raman spectroscopy was used to help in differential diagnosis of malignancy were applied in several kinds of tumors. Cervical cancer is the second most common cancer in women worldwide, and the mortality associated with cervical cancer can be reduced if this disease is detected at the early stages. The results of Lyng et al. (32) show that Raman spectroscopy displays high sensitivity to biochemical changes in tissue during disease progression resulting in excellent accuracy in the discrimination between normal cervical tissue, invasive carcinoma and cervical intraepithelial neoplasia.

The diagnostics of breast cancer was also the subject of a Raman spectroscopy investigation. In the study of Bitar et al. (33), the assignment of the appropriate Raman bands enabled them to connect several kinds of breast tissues, normal and pathological, to their corresponding biochemical moieties alterations and to distinguish among 7 groups: normal breast, fibrocystic condition, duct carcinoma *in situ*, duct carcinoma *in situ* with necrosis, infiltrating duct carcinoma not otherwise specified, colloid infiltrating duct carcinoma, and invasive lobular carcinomas.

Raman spectroscopy continues to push its frontier, enabling individual neoplastic cell identification, such as shown in the works of Chan et al. (34, 35). Owing to improved and advanced Raman techniques,

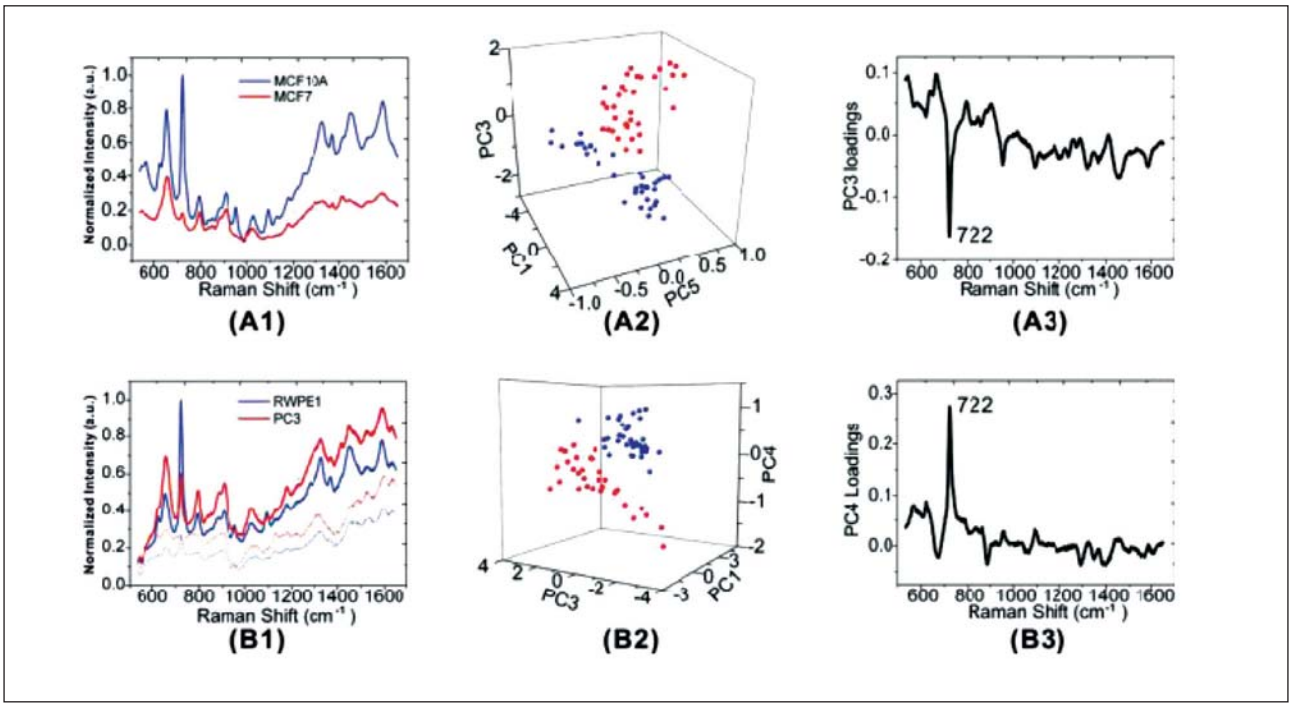

Figure 2 SERS Spectra (A1 and B1), PC plots (A2 and B2), and loading spectra (A3 and B3) of tumor (red) and nontumor (blue) breast (A) and prostate (B) cell lines. Reprinted with the permission from J Phys Chem Lett 2010; 1: 1595–1598. Copyright (2012) American Chemical Society.

they confirmed that it is possible to distinguish normal and neoplastic cells as well.

Cell surface is a very important target in investigations focused on carcinogenesis processes. The work of Bo et al. (36) gave results of exploring the cell surface in cases of breast and prostate cancers, and healthy cells as well, by SERS with Ag nanoparticles as substrate. Obtained spectra differ, especially in the region of 600–900 cm^{-1}. Detection of specific differences in the recorded SERS spectra gave the scientists the clue to introduce biomarkers for tumor cells surface. We found the following example (36) would be illustrative for the wide audience of the journal.

SERS measurements were performed on an upright microscope equipped with a 300 mm focal length imaging spectrometer and a back-illuminated CCD camera optimized for the near-infrared. A 600 lines/mm grating with a blazing wavelength of 750 nm was used. The laser light was injected into the objective and focused onto the sample plane. The laser power at the sample was 21.5 mW. The active area for recording SERS spectra was limited by a slit in the entrance port of the spectrometer to 2 μm x 78 μm. Typically 4–5 cells filled this region. Ten individual acquisitions with a 2 s integration time were accumulated for each spectrum. Each spectrum comes as the average of 36 scans in three independent experiments.

Figure 2 shows examples of the spectra of tumor and nontumor breast and prostate cells. The dashed lines in *Figure 2B1* show the spectra of supernatant prostate cells recorded under the same conditions as the tumor cells SERS spectra background. The differences between the spectra of tumor and nontumor cells are evident. PC plots (so-called scores) were obtained by software simplification of the spectral data (*Figures 2A2* and *2B2*). The PC values for the individual measurements in a data set provide a quantitative measure of the differences between complex spectra. The loading spectra of these PCs are shown in *Figures 2A3* and *2B3*. In both cases, the 722 cm^{-1} band has the highest absolute contribution to the PC. This finding indicates that cancer-specific changes in the cell surface chemistries, which are independent of the cell source (breast or prostate), cause the intensity differences in the 722 cm^{-1} band. The 722 cm^{-1} band arises from molecular species located at the cell surface, so this band was assigned to the C-N bond of quaternary ammonium groups in phosphatidylcholines and sphingomyelins, which are the main components of the membrane lipid bilayer.

Raman Microspectroscopy of Single Cells

The analysis of biological samples and tissues is very complicated, among other things, due to thousands of molecule signals overlapping and the certain possibility that signals with low intensities could be masked if a visible excitation laser source is used. The introduction of an Nd-YAG laser working at 1064 nm grants the application of Raman spectroscopy as a diagnostic tool in different pathologic states (37). The authors gave the Raman microspectroscopy results of individual cells astrocytoma, and some of the representative Raman spectra in the wavenumber range 600–1800 cm^{-1}. The protein bands characteristic of the α helix or collagen helix, DNA bands, and cholesterol bands indicated the differences between healthy and tumor cells.

This demonstrated the applicability of Raman spectroscopy for real-time and *in vivo* diagnosis during neurosurgery. Raman spectroscopic methods might contribute to tumor diagnosis and to defining exact margins intraoperatively, leaving healthy and functional brain tissue intact.

Raman microspectroscopy combines molecular specificity with diffraction-limited resolution in the submicrometer range and can be applied under *in vivo* conditions without fixatives, markers, or stains. After the same steps as explained in the previous example, authors gave the obtained spectra of a dried human osteogenic sarcoma cell (37). The data set was obtained by exciting with a 785 nm diode laser, collecting spectra with 1 min exposure time, and subsequently moving the sample in a raster pattern to map a defined area. Raman bands in raw spectra were assigned to proteins, lipids, and nucleic acids. On the basis of the spectral information the main cellular constituents were highlighted in false color plots. Because image contrast reflects the molecular properties of the specimen, this approach is often called »molecular staining«.

Details of the instrumentation, the analysis, and data from cells in medium have been published elsewhere (38). Similar mapping experiments on fixed single cells have also been described by another group (39). The methodology can even be applied to studies of living cells. It was recently shown for murine lung epithelial cells that their viability was apparently not affected after prolonged irradiation at 785 nm with laser power up to 115 mW, whereas significant morphology changes occurred in cells irradiated with 488 and 514 nm lasers, even with laser power as low as 5 mW (40). Other studies of single cells by Raman spectroscopy involved signal-enhancement methods, for example studies of hemoglobin in single living erythrocytes (41). This special application took advantage of the presence of hemoglobin at high concentrations and the fact that the Raman signals of hemoglobin are enhanced by resonance effects.

Results of a study performed by Wang at al. (42) found protein mucin (MUC4) in considerably high levels in pancreatic adenocarcinoma patients, while vol-

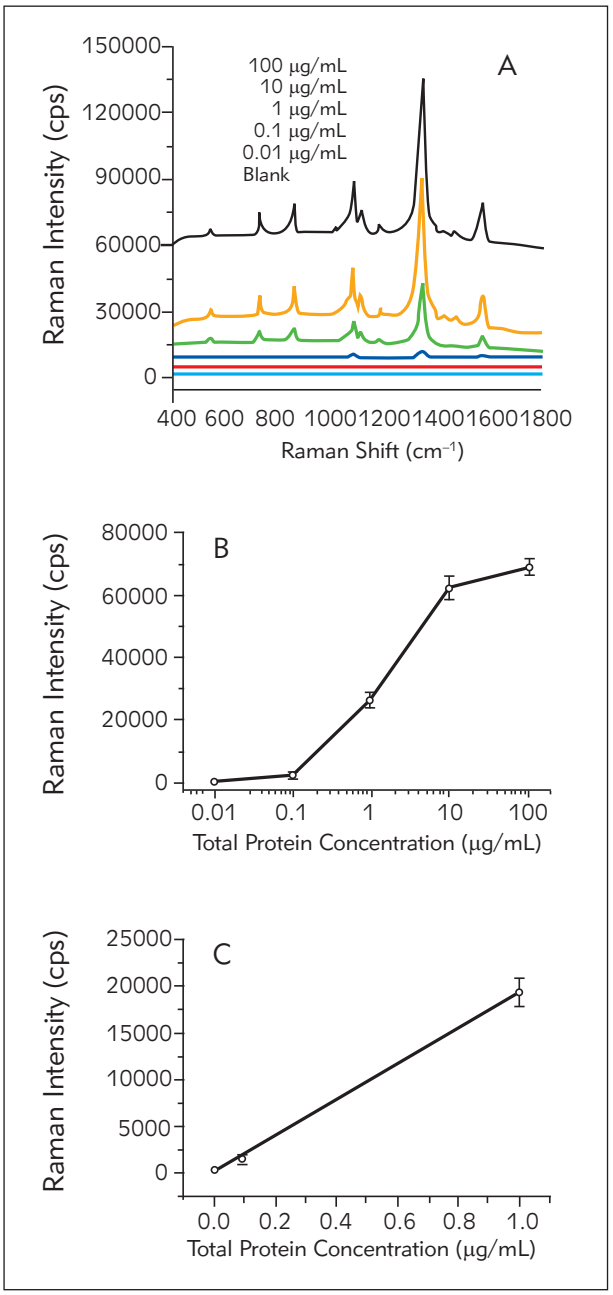

Figure 3 SERS-based assay for MUC4 in PBS buffer. (A) SERS spectra acquired at various CD18/HPAF cell lysate concentrations. (B) Dose-response curve for MUC4 prepared by serially diluting CD18/HPAF cell lysates. (C) Low concentration range on a linear scale. Data points are the average of three separate assays, and the error bars are their standard deviations. Reprinted with the permition from Anal Chem 2011; 83: 2554–2561. Copyright (2012) American Chemical Society.

unteers with normal pancreas and chronic pancreatitis had undetectable levels of MUC4. However, the measurement of MUC4 in sera using conventional test platforms, ELISA and RIA, has been unsuccessful. This has prevented the assessment of the utility of this protein as a possible pancreatic cancer marker in sera.

The Raman spectra were collected with a fiber-optic-based Raman system, a portable, field-deployable instrument. The light source was a 30 mW, 632.8 nm He-Ne laser. The spectrograph consisted of an imaging spectrometer (6–8 cm^{-1} resolution) and a CCD at 0 °C. The incident laser light was focused to a 25 μm spot on the substrate. The analyte concentration was quantified using the peak intensity of the symmetric nitro stretch ($\nu s(NO_2)$) of NTP at 1336 cm^{-1}. Results of SERS-based assays for MUC4 in PBS buffer are shown in *Figure 3* (42).

The team created a simple diagnostic test for MUC4 through the development of a SERS-based immunoassay. The results indicate that a SERS-based immunoassay can monitor MUC4 levels in patient sera, representing a much needed first step toward assessing the potential of this protein to serve as a serum marker for the early stage diagnosis of pancreatic cancer, and demonstrate that the SERS assay outperforms conventional assays with respect to limits of detection, readout time, and required sample volume.

Conclusion

Raman spectroscopy facilitates the determination of the most frequent biochemical parameters, such as glucose, acetone, creatinine, urea, lipid profile, uric acid, and total protein, but also of very specific ones. Development of modern Raman techniques enables the following of the degree and type of atherosclerotic changes in coronary arteries. A broad body of literature deals with the application of Raman spectroscopy in transdermal glucose monitoring with the aim of helping patients with one of the most spread diseases worldwide – diabetes. Valuable information can be obtained for the diagnostics of cancer in its very early stage, and some other diseases could be monitored as well based on the fact that the spectra of healthy/normal cells/tissues and pathologic ones differ according to their chemical characteristics. One of the future goals in this field is developing kits based on data obtained by the Raman spectra.

Thanks to numerous research groups all over the world focusing on further development of Raman spectroscopy, we hope that very soon this method will transform from a »promising analytical tool« to a routine and powerful biochemical technique.

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Conflict of Interest Statement

The authors declare having no conflict of interest related to the publication of this manuscript.

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