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Raman Spectroscopy Applied to Health Sciences

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Abstract

Raman spectroscopy has remarkable analytical abilities to scientists who want to study biological samples. The use of Raman spectroscopy within biologic samples has been increasing in the last years because it can provide biochemical information, allows discrimination between two or more sample groups, and, contrary to what happens with other spectroscopic techniques, water has no interference in the spectra. Biological samples typically do not require extensive preparation, and biochemical and structural information extracted from spectroscopic data can be used to characterize different groups. This chapter presents the general features of Raman spectroscopy and Raman spectroscopic tools relevant to the application in health sciences. In order to emphasize the potential of Raman in this research field, examples of its application in oncology, in bacterial identification and in dementia diagnosis are given.

Keywords: Raman spectroscopy, metabolomics, diagnosis, health and disease

1. Introduction

Spectroscopy is a relevant tool for biomedical analysis. Significant progresses in the application of spectroscopy in clinical field were done in the last years. Spectroscopic techniques provide information at the molecular level, and it is possible to evaluate functional groups, bond types and molecular conformations of the biological components of a sample, once spectral signals in vibrational spectra are specific to each molecule and act like a fingerprint.

A spectroscopic-based metabolomic study includes sample collection, sample analysis, statistical analysis and identification of altered metabolites. The resulting data can be translated into defining disease biomarkers/pathways, with the generation of a disease metabolic fingerprint.

Vibrational spectroscopy has been proposed as an approach to diagnosis. Raman has a past research regarding its potential as a diagnostic tool of a wide range of pathologies using a

wide range of sample types. Although being recognized as not as specific and sensitive as other metabolomics techniques, several works already demonstrated the potential of Raman applied to health sciences for metabolic fingerprinting because it is possible with only one spectra to simultaneously analyze carbohydrates, amino acids, fatty acids, lipids, proteins, nucleic acids and polysaccharides with a minimum sample preparation. As Raman is a scattering technique and it is not perturbed by aqueous media, it is suitable to analyze biological samples.

For diagnostic purposes, it is expected that Raman spectra of biological samples result in quantitative data, so it is essential to define some categorical differentiable classes for data by dividing samples in healthy or disease sample classes. For these purposes, chemometric data processing is a valuable tool.

Due to Raman spectroscopy features, it is currently widely used in health sciences for spectral imaging of cells and tissues, for the *in-vivo* and *ex-vivo* diagnosis of tissues, where fiber probes can be used, and for biofluid analysis, contributing to a better knowledge of the disease and disease diagnosis at the molecular level. This chapter describes the most relevant application of Raman in biomedical field.

2. Raman features and sampling

Raman spectroscopy is an optical technique based on inelastic scattering of light due to the vibration of the molecules that can provide chemical fingerprints of several samples. In health sciences, Raman can be used in DNA analysis, lipids, proteins and amino acids identification, bacteria classification and recognition, cell responses, cancer diagnosis and prognosis, and dental prosthesis, among others. This wide range of application is due to Raman's capabilities that go far beyond of being a noninvasive and nondestructive method that does not require samples preparation. Raman is compatible with aqueous solutions and is also a technique that produces results in a short time, requiring less than a minute to obtain a good quality spectrum, and no sample preparation is needed. This spectroscopic technique is sensitive to identify many different functional groups that produce weak signals in infrared spectroscopy (C=C; S—S; C—S). Besides that, it has a highly selective fingerprint that allows the discrimination of similar molecules and has high spatial resolution that allows single cell analysis and intracellular imaging.

The ability to use advanced optical technologies in the visible or near-infrared spectral range (lasers, microscopes, and optic fibers) is responsible for the growth of Raman spectroscopy in medical diagnostic. As molecular changes in cells, tissues or biofluids can be detected and quantified by Raman spectroscopy, it is possible to use this technique in the diagnosis of diseases and to study the effects of drugs (treatments) in biosystems.

In fact, Raman spectroscopy can offer chemical fingerprints of cells, tissues (*in vivo* or *in-vitro*), or biofluids. A large range of samples can be used for Raman analysis: formalin-fixed and fresh frozen mammalian tissue, fixed cells and biofluids.

Whether a sample is a solid, liquid, powder, slurry, or gas, no sample preparation is normally required, and there is no need to dissolution, grinding, glass formation, or pressing in order

to obtain good spectroscopic outcome. Raman spectroscopy is also a nondestructive method, and it is possible to evaluate the chemical composition of a sample, and the same aliquot can be used further to extract additional biological information by using other methodologies [1].

3. Raman spectroscopic variations

In recent past, Raman spectroscopy was known as a technique with low signals requiring longer acquisition times. However, recent developments were made to overcome this limitation in the last years. Nonlinear optical effects and metallic nanoparticles are currently used to improve Raman signals, fiber-optic Raman probes were introduced and are used for real-time *in-vivo* experiments, and multimodal integration with other optical techniques increased the acquisition speed and spatial accuracy. These advances in the accuracy allow the application of Raman spectroscopy into clinical diagnosis, and time of analysis allows its clinical use.

Surface enhanced Raman spectroscopy (SERS), Tip Enhanced Raman Scattering (TERS) or nano-Raman and resonance effects increase significantly Raman sensitivity to study biological samples. SERS increases Raman intensity compared to the usual and weak Raman scattering. These improvement features are sufficient to allow even single molecule detection using Raman. SERS is useful in trace material analysis, flow cytometry and other applications where the traditional sensitivity/speed of a Raman measurement is insufficient [2]. Resonance Raman spectroscopy is a variant of Raman spectroscopy that instead of using laser excitation at any wavelength to measure Raman scattering of the laser light, the excitation wavelength is used to overlap with an electronic transition. The overlap results in an extraordinary increase in scattering intensities, thus detection limits and measurement times can be significantly decreased. It is also possible to couple Raman to an optical microscope. Raman microspectroscopy uses visible and near-infrared excitation lasers and allows to extract molecular properties of the samples with diffraction-limited spatial resolution. The typical method to obtain Raman spectral images is by scanning the sample with the laser spot and then applying a uni- or multivariate spectral model to each Raman spectrum [3]. In order to decrease the time of analysis, Raman spectral imaging can be based on line-mapping (laser beam is expanded to form a line spot on the sample surface) [4].

TERS is a chemical imaging technique that is label-free and have enhanced-resolution. TERS imaging is performed with a Raman spectrometer, a scanning probe microscope (SPM) integrated with an optical microspectrometer. The scanning probe microscope provides the means for nanoscale imaging and the optical microscope provides the resources to bring the light to a functionalized probe, and the spectrometer is the sensor analyzing the light output providing chemical specificity. It is possible to increase the signal to obtain high-spatial resolution spectral images for large samples using selective-sampling Raman microspectroscopy. In this approach, it is possible to (1) obtain information about sample spatial features by other optical technique [5] or (2) estimate information in real-time from the Raman spectra [6]. When traditional variations of Raman spectroscopy are used to study tissues, the results are not good due to insufficient penetration depth. The advance of spatially offset Raman spectroscopy (SORS) overcame this limitation enabling spectral measurements until 10–20 mm of

the sample and, with this modification, the application of Raman in clinical fields increases by collecting the scattered light away from the point of laser illumination [7]. Transmission Raman spectroscopy (TRS) is the term used when the collection and illumination points are on opposite sides of the sample, and it is quite useful to analyze opaque materials. Besides, it was proved to be very useful to read many millimeters of tissues [8]. To increase the resolution of Raman microspectroscopy, it is possible to use Coherent anti-Stokes Raman spectroscopy (CARS) or stimulated Raman spectroscopy (SRS), both based on nonlinear optical effects that upgrade the spatial resolution. Coherent Raman spectroscopy techniques are based on non-linear effects to increase the speed and spatial resolution of Raman spectroscopy by inducing coherent molecular vibrations in the sample increasing resolution. CARS is used to obtain image from cells and tissues by exciting the CH stretching vibrations of lipids and proteins and SRS allows to obtain high-speed images [9, 10]. Raman spectroscopy is also adaptable for fiber-optic probes, making it valuable for medical diagnosis *in vivo*, for instance in hollow organs. The probe should have very reduced dimensions to permit the access to body cavities, and the spectra acquisition time should be short to allow accurate measurement [11].

4. Multivariate analysis applied to spectroscopic data analysis

Multivariate analysis tools are used to extract information from spectral data set and to assist in biomedical interpretation. Chemometric methods are diverse and offer different approaches to extract specific information from the data. These methods used for data interpretation and to extract information from complex datasets were usually univariate and therefore they were not suitable to describe the sample variation or composition. A range of processing methods can be applied to a Raman data set; usually baseline corrections should be performed as a first approach, prior to normalization methods and then a wide range of multivariate analysis tolls can be applied [12].

Multivariate methods include techniques of multivariate classification (or pattern recognition techniques) and multivariate regression. Techniques of multivariate classification can be divided into unsupervised and supervised learning procedures. In unsupervised pattern recognition techniques such as principal-component analysis (PCA) and cluster analysis (CA), there is no need for *a priori* knowledge about the training set samples (spectra). These methods are used to consider differences and similarities between spectra. By contrast, supervised pattern recognition techniques such as linear discriminant analysis (LDA) and artificial neural networks (ANNS) require some *a priori* knowledge, for example, undoubtedly identifying samples from disease and samples from healthy cases. In this way, supervised procedures allow a more precise classification, while unsupervised methods are useful for an exploratory analysis of data.

On the other hand, multivariate regression techniques (or multivariate calibration methods) are usually applied to analyze one or multiple molecules of a complex sample that possess overlapping spectroscopic signals. These techniques include principal components regression (PCR) and partial least squares regression (PLS) (**Figure 1**).
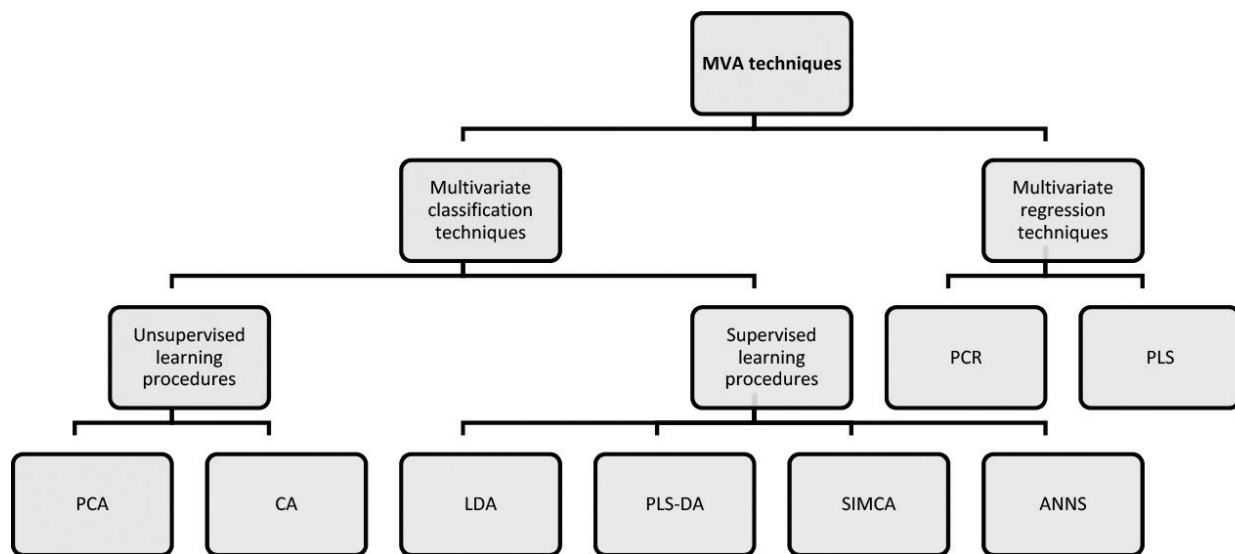
Figure 1. Most frequently applied multivariate data analysis techniques in combination with spectroscopic methods. MVA: Multivariate data analysis; PCR: Principal component regression; PLS: Partial least-squares regression; PCA: Principal-component analysis; CA: Cluster analysis; LDA: Linear discriminant analysis; PLS-DA: Partial least-squares discriminant analysis; SIMCA: Soft independent modeling of class analogy; ANNS: Artificial neural networks. Adapted from [13, 14].

5. Raman spectroscopy in cancer diagnosis

A preeminent application of Raman spectroscopy in health sciences is its use in cancer diagnosis. According to Cancer Research UK, in 2012, an estimated 14.1 million new cancer cases occurred worldwide, resulting in 8.2 million people died [15]. Despite the majority of new cancer cases was registered in less-developed countries, a significative number (more than 6 million cases) occurs in developed regions, with access to advanced medical care and treatments [16]. These data reveal the urgent need of reliable diagnostic tools to reduce cancer cases and cancer mortality.

The common methodologies for cancer diagnosis are based on invasive histological analysis and biomedical imaging, which are expensive, time-consuming and can give rise to subjective diagnosis [17]. Raman spectroscopy has been extensively studied in an attempt to replace or complement current methods and increase sensitivity and specificity of the diagnosis [18]. In this section, we discuss the advances in Raman spectroscopy applied to biofluids and tissue for diagnosis of different cancer types (**Figure 2**).

5.1. Skin cancers

Raman has been widely investigated to discriminate benign skin lesions from malignant lesions, mainly basal cell carcinoma and squamous cell carcinoma (**Table 1**). Despite the high complexity of skin tissue, studies *in vivo* show huge potential as a routine procedure in hospitals for cancer screening [19]. In 2008, a preliminary report used 289 patients with different
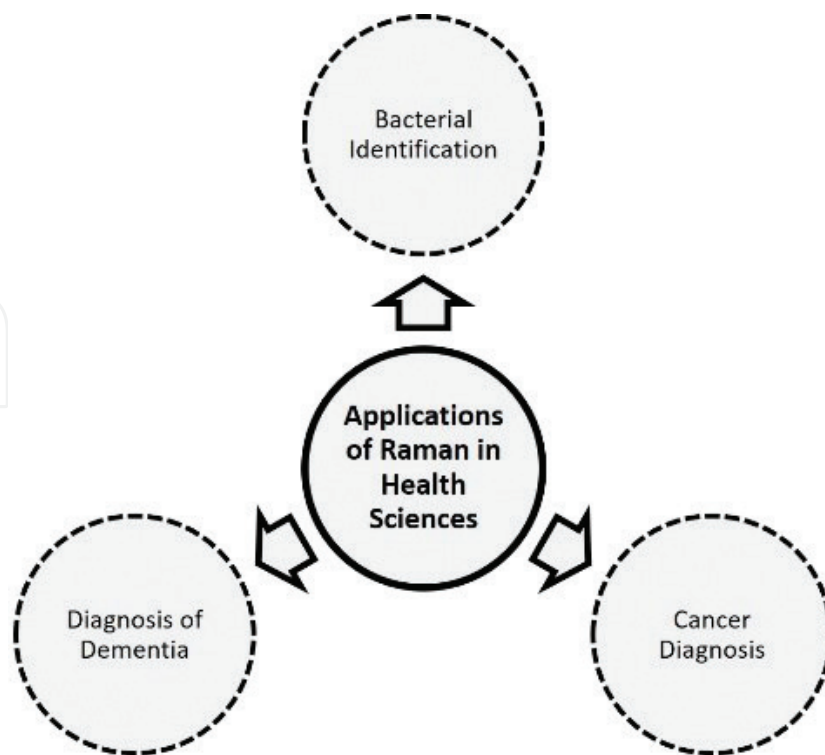
Figure 2. Main application areas of Raman spectroscopy in health science.

types of malignant and benign skin lesions [20]. The authors applied real-time *in vivo* Raman spectroscopy to acquire spectra of all samples and analyzed data using PLS and LDA analysis. Direct analysis of spectra showed distinct biomolecular signatures and ROC curves allowed to discriminate skin cancers from benign lesions with an area under curve greater than 0.9 [20]. Later, the same authors published another study *in vivo* using a higher cohort of patients and 518 skin lesions (both malignant and benign conditions that are visually similar to skin cancer) [21]. Using the same instrumentation as before [20], the authors analyzed data using PC-GDA and PLS analysis, once visual inspection of spectra did not show distinctive Raman peaks assigned to skin cancer [21]. Although Raman peaks are almost the same between normal and cancer samples, the intensity of the signals allows the discrimination between different types of lesions and are compatible with those obtained using histopathological methods. To complement and validate these results, in 2015, Zhao et al. [22] added 127 samples to the previous cohort, creating a consolidated group. Using real-time Raman spectroscopy and PC-GDA, an PLS analysis obtained the same discrimination as before, proving the ability of Raman spectroscopy for *in vivo* skin cancer detection. In 2014, other *in vivo* study successfully used Raman microspectroscopy to study 20 skin samples and detect basal cell carcinoma in tissue removed during surgery [21].

Besides *in vivo* studies, there are also some studies *in vitro* to assess the use of Raman spectroscopy for skin cancer detection. In 2010, Bodanese et al. used dispersive Raman spectroscopy and successfully distinguished normal samples from basocellular cell carcinoma [23]. They found spectral differences in the region between 800 and 1000 cm^{-1} and between 1200 and

Cancer type	Study type	Statistical analysis	Sensitivity and specificity (%)	Spectral differences (cancer *vs.* normal)	Refs.
Skin	*In vivo*	PC-GDA, PLS	95, 54	NA	[22]
Skin	*Ex vivo*	LDA, MLR	100, 92	NA	[44]
Skin	*Ex vivo*	PCA	99.1, 93.3	Proteins, lipids and melanin	[24]
Skin	*In vitro*	GDA	~90, ~90	Phenylalanine, tryptophan and DNA bases	[25]
Skin	*In vivo*	PC-GDA, PLS	90, 64	NA	[21]
Skin	*In vitro*	PCA	89, 93	Lipids and proteins	[23]
Skin	*In vivo*	PLS, LDA	91, 75	NA	[20]
Nasopharyngeal	*In vivo*	PLS	91, 95	NA	[33]
Oral	*Ex vivo*	PCA, LDA	80.7, 84.1	Nucleic acids, proteins, lipids	[28]
Laryngeal	*In vivo*	PLS-DA, LOPCV	93.3, 90.1	Nucleic acids, proteins, lipids	[32]
Esophageal	*In vivo*	PLS-DA, LOPCV	97, 97.4	Lipids, proteins	[31]
Esophageal	*Ex vivo*	PCA	90.5, 95	NA	[45]
Esophageal	*Ex vivo*	PCA, LDA	93, 95	NA	[30]
Esophageal	*Ex vivo*	PCA, LDA	71–81, 81–98	NA	[29]
Nasopharyngeal	*Ex vivo*	PCA, LDA	90.7, 100	Nucleic acids, collagen, phospholipids, phenylalanine	[27]
Gastric intestinal pre-cancer	*In vivo*	PCA, LDA, LOPCV	89.3, 92.2	Collagen, lipids, phenylalanine, proteins	[36]
Gastric	*Ex vivo*	PCA-DA	90, 90.9	NA	[46]
Gastric	*Ex vivo*	PCA, LDA	100, 94.1	RNA bases, ribose	[35]
Gastric	*Ex vivo*	PCA, LDA	100, 97	Tyrosine, adenine, coenzyme A	[34]
Breast	*Ex vivo*	Direct analysis	NA	Carotenoids, lipids, carbohydrates, proteins	[40]
Breast	*Ex vivo*	PLS-DA, LOPCV	74.2, 86.4	Collagen, amino acids	[39]
Breast	*Ex vivo*	LDA, MNLR	95.6, 96.2	Nucleic acids, collagen, lipids	[38]
Leukemia	*Ex vivo*	PCA, LDA	100, 100	Lipids, phospholipids, amino acids, carotenes	[43]
Colorectal	*Ex vivo*	PCA, LDA	97.4, 100	Nucleic acids, saccharides, proteins	[42]
Bladder	*In vitro*	PCA	98, 95	NA	[41]

GDA: General discriminant analysis; LDA: Linear discriminant analysis; LOPCV: Leave-one patient-out cross validation; MLR: Multinomial logistic regression; MNLR: Multinomial logistic regression; NA: Not available; PCA: Principal component analysis; PC-GDA Principal component with generalized discriminant analysis; PLS: Partial least square; PLS-DA: Partial least square discriminant analysis.

Table 1. Raman spectroscopy in cancer diagnosis.

1300 cm^{-1}, assigned to proteins and lipids, using PCA multivariate analysis, with high sensitivity and specificity [23]. Later, in 2012, the same author reported new results corroborating the previous study [24]. Using a Raman spectrometer attached to a fiber optic and PCA analysis, they analyzed 145 different samples of basocellular cell carcinoma, melanoma and without malignant lesions and were able to discriminate cancer and normal samples with sensitivity and specificity values over 90% [24]. In the same year, a different study was performed by Wang H et al. using HaCaT cells, melanocytes and their malignant derivatives [25]. They tested the ability of micro-Raman spectroscopy to separate different cell lines and found significant spectral differences between HaCaT cells and squamous cell carcinoma, melanocytes and melanoma cells as well as between all normal cells *versus* all tumor cells [25] (**Table 1**). The results of these *in vitro* studies are of extreme importance and can help the interpretation spectra of *in vivo* samples for cancer skin diagnosis.

5.2. Oral cavity, nasopharyngeal and laryngeal cancers

In what concerns diagnostic of oral cancers, Raman spectroscopy has been widely used in biofluids like blood, urine and saliva and using fiber optic probes for *in vivo* diagnosis (**Table 1**). The advantage of using biofluids instead of tissue is that they can be collected using noninvasive and painless methods [26].

In 2010, Feng et al. described for the first time the use of a surface-enhanced Raman spectroscopy (SERS) method for plasma analysis for nasopharyngeal cancer detection using silver nanoparticles [27]. This type of spectroscopy produces strong signals and has a low associated cost. Besides, it is suitable for use with intact tissue, biofluids and during endoscopies [26]. A total of 76 samples were used in that study and using PCA and LDA multivariate analysis, the authors found a distinct biological signature between control and cancer samples mainly due to an increase in nucleic acids, collagen, phospholipids and phenylalanine and also a decrease in amino acids and saccharide in cancer samples compared to control samples [27]. These results gave new insights about the use of surface-enhanced Raman spectroscopy for noninvasive diagnostic methods. SERS using gold nanoparticles was also applied to blood serum to diagnose oral squamous cell carcinoma [28]. Analysis of 370 Raman spectra using PCA and LDA multivariate analysis allowed to discriminate serum samples of patients with and without neoplasia with high sensitivity and specificity [28].

The use of Raman spectroscopy in oral cancer diagnosis relies mainly on fiber optic probes, and data published so far present promising results. In 2010, a study showed the applicability of fiber optic Raman probed spectroscopy to target biopsies at endoscopy [29]. This type of approaches can be useful to avoid excision of normal tissue for biopsy. In this way, there is a reduction of the burden in histopathology departments and in the number of invasive procedures for the patient. Later, in 2012, Almond et al. also tested a fiber optic Raman probe to discriminate between benign, metaplastic and neoplastic esophageal *ex vivo* tissue [30]. Results showed high sensitivity and specificity, so it is suitable to think in this approach as a new technique for clinical diagnosis although *in vivo* clinical trials are needed to confirm the accuracy of this probe. In 2015, fiber optic Raman spectroscopy was successfully applied in 48 patients during endoscopy [31]. The authors were able to simultaneously acquire both

fingerprint and high wavenumber Raman spectra to extract the maximum biological information and obtained sensitivity and specificity values about 97% for the diagnosis of esophageal squamous cell carcinoma [31]. Similar results were obtained with a probe designed to diagnose laryngeal cancer [32]. Analysis of 2124 Raman spectra of 60 patients during endoscopy showed sensitivity and specificity above 90% for the identification of laryngeal cancer when combined fingerprint and high-wavenumber spectra [32]. Recently, Ming et al. [33] performed a pilot study in 79 patients with and without nasopharyngeal cancer and in post-irradiated patients. They detect a specific signature for each one of the three cohorts, which may indicate that Raman could not only be used for diagnostic purposes but also for surveillance in post-treated patients. Furthermore, the authors used a probe with only 1.8 mm, which is the smallest probe used in Raman diagnostics and is more suitable to be used in clinical endoscopies [33].

5.3. Gastric cancers

Diagnosis of gastric cancer using Raman technologies relies either on the use of fiber optic probes or on SERS (**Table 1**). In fact, SERS was applied to plasma samples to detect gastric cancer in a noninvasive way [34], similar to what was done to diagnose nasopharyngeal cancer [27]. The authors use two cohorts, with a total of 65 samples (32 patients with confirmed gastric cancer and 33 control patients). Using PCA and LDA multivariate analysis, it was observed discrimination between cancer and normal samples with sensitivity and specificity of 100 and 97%, respectively. In 2012, the same methodology was applied to discriminate gastric cancer from normal controls based on serum RNAs, also achieving high sensitivity and specificity (100 and 94.1%, respectively) [35]. SERS seems to be a useful technique to apply in routine clinical diagnosis coupled, for instance, with endoscopy. Fiber optic probes can be used for *in vivo* identification of gastric metaplasia. Lin et al. [36] coupled fingerprinting and high-wavenumber Raman spectroscopy with a fiber optic Raman probe and were able to detect, in real-time, pre-cancerous gastric lesions. They acquired 4520 spectra in real time, during gastroscopy, and by using PCA and LDA analysis, they were able to identify precancerous lesions with high sensitivity and specificity [36]. This can improve early diagnosis of neoplasia and significantly improve the efficacy of treatments.

5.4. Breast cancers

Breast cancer is the second most prevalent cancer in the world and, it is the most common cancer in women, causing more than 500,000 deaths every year [37]. According to these statistics, it is not surprising that Raman spectroscopy has been used as a diagnostic tool for this disease. Kong et al. used Raman microspectrometry to detect ductal carcinoma in tissue excised during breast-conserving surgery [38]. They developed a model that allowed to discriminate normal and cancerous tissue in approximately 17 min, with sensitivity and specificity above 95% [38]. A different approach was used by Feng et al. in 2015 [39]. Similar to what this group did for other types of cancer (see **Table 1** for detailed information), they applied SERS to saliva proteins of 97 patients and were able to discriminate between control, benign tumors and malignant tumors with sensitivities and specificities between 72.7–75.8% and 81.2–93.4%, respectively, using PLS-DA analysis [39]. These results give good perspectives for new

noninvasive diagnostic tools. Depciuch et al. acquired Raman spectra of breast biopsies and did direct spectral analysis without performing multivariate analysis [40]. They reported differences in spectral regions assigned to the principal biomolecules: lipids, sugars and proteins between normal and cancerous samples [40]. All these reports give new light in understanding the molecular mechanisms involved in breast cancer.

5.5. Other cancers

Besides skin, oral, gastric and breast cancers, Raman spectroscopy techniques are also applied to diagnose other types of neoplasia (**Table 1**), mainly *in vitro*. For instance, in 2011, there was a study that used modulated Raman spectroscopy to detect the presence of human urothelial cells and bladder cancer cells after cell lines were incubated with urine [41]. The results achieved high sensitivity and specificity and, in the future, this approach may be applied in routine urine exams to detect bladder cancer or to monitor patients under treatment for bladder cancer.

Blood samples are widely used in the context of diagnosis. In the case of colorectal cancer, Lin et al. used gold nanoparticle-based SERS in blood serum samples of 83 patients to differentiate Raman spectra of healthy and disease samples [42]. This approach allowed to achieve sensitivity and specificity of 97.4 and 100%, respectively. Besides, the authors detected spectral differences between normal and cancer samples, mainly an increase in the relative amount of nucleic acids and a decrease in the amount of proteins in colorectal cancer patients, compared to control healthy subjects [42]. Serum blood samples can also be used to monitor the efficacy of treatments. In the specific case of leukemia, Gonzalez et al. used standard Raman spectroscopy and multivariate analysis to distinguish normal samples from leukemia samples with 100% of both sensitivity and specificity [43]. The authors detect some molecular changes between both groups of samples, mainly in the regions of lipids, phospholipids and β-carotene. Therefore, this Raman-PCA technique can be easily applied as a noninvasive tool to diagnosis and progression evaluation of leukemia.

In the last decade, there was an increase in the use of Raman spectroscopy in the field of cancer diagnostic and monitoring. As it was possible to see in this section, the improvement in the algorithms to process Raman signals as well as the development of new SERS techniques and fiber optic probes allowed to produce results with high sensitivity and specificity and to apply Raman-based approaches in *in vivo*, *ex vivo* and *in vitro* clinical diagnosis of several cancer types in different biological samples.

6. Raman spectroscopy for bacterial identification

Correct and in-time identification of microorganisms is crucial in clinical diagnosis. Nowadays, despite the advances of technology and methodologies of bacterial identification, most of the hospitals use bacterial culture as a standard method [47, 48]. However, these approaches are time-consuming, and sometimes it requires more than a day until the results are available. This can have serious implications to patients, mainly to those with severe infections. In this way, there are several investigations that evaluate the potential of Raman-based approaches

to bacterial identification and typing and producing fast and accurate results. Raman spectra of bacteria are like a fingerprint, since it represents the molecular composition and it is specific for each sample. The following studies are examples of the use of Raman in the field of clinical microbiology.

In 2009, Willemse et al. used Raman spectroscopy to type methicillin-resistant and methicillin-sensible *Staphylococcus aureus* and compare it with traditional DNA typing methods, which are time- and labor-consuming [49]. After spectra collection and cluster analysis, results showed that Raman spectroscopy has reproducibility and discrimination ability, and all Raman clusters were in accordance with epidemiologic data of the isolates [49]. Similar results were obtained by the same group using *Escherichia coli* and *Klebsiella pneumoniae* isolates [50]. Using SpectraCell analyzer (River Diagnostics), they obtained high reproducible spectra and a discriminatory power similar to traditional DNA typing methods [50]. However, Raman spectroscopy was not able to detect ESBL-producing *E. coli* transmission events even when coupled to High-throughput MultiLocus Sequence Typing [51]. SERS is also used in the field of microbiology to identify pathogens. For instance, malaria parasite can be detected using a SERS nanoplatform [52]. Besides, it is also possible to discriminate wild-type malaria DNA from mutant malaria DNA using this technique [52]. SERS was also used in milk samples to identify *Salmonella enterica* serotype Enteritidis [53]. In this study, SERS was used with Au nanoprobes, and the results were almost 100x more sensitive than those obtained by PCR [53].

In clinical microbiology, it would be of particular interest to apply bacterial identification approaches directly to biological samples. One of the possible drawbacks of using spectroscopy in this field is that biological fluids can have complex matrixes that may mask the specific spectral signature of a given pathogen. To verify the applicability of Raman to bacterial identification in biofluids, Harz et al. used micro-Raman to directly analyze cerebrospinal fluid (CFS) of patients with bacterial meningitis [54]. Since lethality of the disease depends on the pathogen involved, time is crucial and it is necessary to properly identify the bacteria and initiate the adequate antibiotic therapy in a short period of time. In this study, the authors showed that CFS did not affect Raman spectra of bacteria, and it was possible to identify it with accuracy [54]. This corroborates the idea that Raman can be used as a diagnostic assay. In hospitals, it is important not only to identify the pathogen but also to understand the antimicrobial susceptibility profile of the microorganism in order to choose the right antibiotic to mitigate and treat the infection. Raman spectroscopy has been recently successfully used with this purpose [55]. The authors were able to discriminate Raman spectra of 67 antibiotic-susceptible strains isolated from positive blood cultures in the presence of different concentrations of antibiotic in only 5 h [55]. Further development of this technology could produce results with robustness similar to current methods used in hospitals, and therefore in the future, it can be applied to clinical diagnosis.

Raman-based approaches can also be used in the field of virology. It is possible to identify rotavirus with an accuracy above 96% using SERS fingerprinting, and the detection of the virus was possible even using a complex cellular matrix, although the results were not as sensitive as those obtained with purified samples [56]. A similar procedure was used to detect respiratory syncytial virus [57]. In this study, the authors applied SERS enzyme-catalyzed immunoassay of respiratory syncytial virus in cell lysates, and the results showed a linear

correlation between the intensity of spectra and the amount of virus with a detection limit lower than traditional methods [57].

The studies discussed in this section suggest that Raman-based approaches are a good alternative for real-time clinical usage, since they are easy to use, fast (it is possible to have results within 45 min after positive culture), reliable and can be easily applied to a wide variety of microorganisms, since specific dyes or labels are no need.

7. Raman spectroscopy for the diagnosis of dementia

Alzheimer's disease is the most prevalent type of neurodegenerative dementia in the elderly population worldwide. The key for efficiently and accurately diagnosing dementia occurs during the early stages. Scientists struggle to find a blood-based method to perform an accurate diagnosis of this type of dementia, which affects 35.6 million people worldwide [58].

In this context, Raman microspectroscopy was already used to analyze serum of Alzheimer's disease patients, patients with other dementias and healthy controls. Data were analyzed with multivariate statistics for the differential identification of Alzheimer's disease patients. The study was a proof of concept; however, it proves that Raman microspectroscopy and artificial neural network classification were able to differentiate patients with more than 95% sensitivity and specificity, demonstrating that Raman has potential to become a blood-based tests that may help clinical assessments for effective and accurate differential diagnosis of Alzheimer's disease [59].

8. Conclusions and outlook

In the last years, there was a significant improvement in instrumentation of Raman spectroscopy, leading to an enhancement of sensitivity and accuracy of this approach. In this way, Raman has been broadly used in the context of medical investigation, mainly in the oncologic field. Coupled to adequate chemometric analysis tools, Raman spectroscopy, mainly SERS, was already applied to successfully diagnose several types of cancer, infections and preliminary results are also promising in the context of Alzheimer's disease. It is expected that in the next years, Raman spectroscopy can be routinely used in hospitals to *in vivo* diagnose and surveillance of several diseases, producing fast and accurate results, decreasing waiting times and, in this way, improving efficacy of treatments.

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References

[1]   Kong K, Kendall C, Stone N, Notingher I. Raman spectroscopy for medical diagnostics — From in-vitro biofluid assays to in-vivo cancer detection. Advanced Drug Delivery Reviews. 2015;**89**:121-134. DOI: 10.1016/J.ADDR.2015.03.009

[2]   Schütz M, Steinigeweg D, Salehi M, Kömpe K, Schlücker S. Hydrophilically stabilized gold nanostars as SERS labels for tissue imaging of the tumor suppressor p63 by immuno-SERS microscopy. Chemical Communications. 2011;**47**:4216-4218

[3]   Delhaye M, Dhamelincourt P. Raman microprobe and microscope with laser excitation. Journal of Raman Specroscopy. 1975;**3**:33-43

[4]   De Grauw CJ, Otto C, Greve J. Line-scan Raman microspectrometry for biological applications. Applied Spectroscopy. 1997;**51**:1607-1612

[5]   Rowlands CJ, Varma S, Perkins W, Leach I, Williams H, Notingher I. Rapid acquisition of Raman spectral maps through minimal sampling: Applications in tissue imaging. Journal of Biophotonics. 2012;**5**:220-229. DOI: 10.1002/jbio.201100098

[6]   Kong K, Rowlands CJ, Varma S, Perkins W, Leach IH, Koloydenko AA, et al. Increasing the speed of tumour diagnosis during surgery with selective scanning Raman microscopy. Journal of Molecular Structure. 2014;**1073**:58-65

[7]   Matousek P, Clark IP, Draper ERC, Morris MD, Goodship AE, Everall N, et al. Subsurface probing in diffusely scattering media using spatially offset Raman spectroscopy. Applied Spectroscopy. 2005;**59**:393-400

[8]   Matousek P, Parker AW. Bulk Raman analysis of pharmaceutical tablets. Applied Spectroscopy. 2006;**60**:1353-1357

[9]   Saar BG, Freudiger CW, Reichman J, Stanley CM, Holtom GR, Xie XS. Video-rate molecular imaging in vivo with stimulated Raman scattering. Science (80-). 2010;**330**:1368-1370

[10] Evans CL, Potma EO, Puoris' haag M, Côté D, Lin CP, Xie XS. Chemical imaging of tissue in vivo with video-rate coherent anti-stokes Raman scattering microscopy. Proceedings of the National Academy of Sciences of the United States of America. 2005;**102**:16807-16812

[11] Matousek P, Stone N. Recent advances in the development of Raman spectroscopy for deep non-invasive medical diagnosis. Journal of Biophotonics. 2013;**6**:7-19. DOI: 10.1002/jbio.201200141

[12] Afseth NK, Segtnan VH, Wold JP. Raman spectra of biological samples: A study of preprocessing methods. Applied Spectroscopy. 2006;**60**:1358-1367

[13] Wentzell PD, Montoto LV. Comparison of principal components regression and partial least squares regression through generic simulations of complex mixtures. Chemometrics and Intelligent Laboratory Systems. 2003;**65**:257-279

[14] Ellis DI, Goodacre R. Metabolic fingerprinting in disease diagnosis: Biomedical applications of infrared and Raman spectroscopy. Analyst. 2006;**131**:875-885. DOI: 10.1039/b602376m

[15] Cancer Research UK. Worldwide Cancer Statistics n.d. Available from: http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer [Accessed: Aug 21, 2017]

[16] Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012: Estimated cancer incidence, mortality and prevalence worldwide in 2012: IARC CancerBase No. 11 International Agency for Research on Cancer 2014. Available from: http://globocan.iarc.fr [Accessed: Mar 16, 2016]

[17] Almond LM, Hutchings J, Shepherd N, Barr H, Stone N, Kendall C. Raman spectroscopy: A potential tool for early objective diagnosis of neoplasia in the oesophagus. Journal of Biophotonics. 2011;**4**:685-695. DOI: 10.1002/jbio.201100041

[18] Pence I, Mahadevan-Jansen A. Clinical instrumentation and applications of Raman spectroscopy. Chemical Society Reviews. 2016;**45**:1958-1979. DOI: 10.1039/C5CS00581G

[19] Kourkoumelis N, Balatsoukas I, Moulia V, Elka A, Gaitanis G, Bassukas ID. Advances in the in vivo Raman spectroscopy of malignant skin tumors using portable instrumentation. International Journal of Molecular Sciences. 2015;**16**:14554-14570. DOI: 10.3390/ijms160714554

[20] Zhao J, Lui H, McLean DI, Zeng H. Real-time raman spectroscopy for non-invasive skin cancer detection - preliminary results. Vol. 2008, 30th Annual International Conference of the IEEE Engineering in Medicine and Biology Society, Vancouver, BC. 2008; pp. 3107-3109. DOI: 10.1109/IEMBS.2008.4649861

[21] Lui H, Zhao J, McLean D, Zeng H. Real-time Raman spectroscopy for in vivo skin cancer diagnosis. Cancer Research. 2012;**72**:2491-2500. DOI: 10.1158/0008-5472.CAN-11-4061

[22] Zhao J, Lui H, Kalia S, Zeng H. Real-time Raman spectroscopy for automatic in vivo skin cancer detection: An independent validation. Analytical and Bioanalytical Chemistry. 2015;**407**:8373-8379. DOI: 10.1007/s00216-015-8914-9

[23] Bodanese B, Silveira LJ, Albertini R, Zangaro RA, Pacheco MTT. Differentiating normal and basal cell carcinoma human skin tissues in vitro using dispersive Raman spectroscopy: A comparison between principal components analysis and simplified biochemical models. Photomedicine and Laser Surgery. 2010;**28**(Suppl 1):S119-S127. DOI: 10.1089/pho.2009.2565

[24] Bodanese B, Silveira FL, Zangaro RA, Pacheco MTT, Pasqualucci CA, Silveira LJ. Discrimination of basal cell carcinoma and melanoma from normal skin biopsies in vitro through Raman spectroscopy and principal component analysis. Photomedicine and Laser Surgery. 2012;**30**(7):381. DOI: 10.1089/pho.2011.3191

[25] Wang H, Tsai T-H, Zhao J, Lee AMD, Lo BKK, Yu M, et al. Differentiation of HaCaT cell and melanocyte from their malignant counterparts using micro-Raman spectroscopy

guided by confocal imaging. Photodermatology, Photoimmunology & Photomedicine. 2012;**28**:147-152. DOI: 10.1111/j.1600-0781.2012.00660.x

[26] Austin LA, Osseiran S, Evans CL. Raman technologies in cancer diagnostics. Analyst. 2016;**141**:476-503. DOI: 10.1039/c5an01786f

[27] Feng S, Chen R, Lin J, Pan J, Chen G, Li Y, et al. Nasopharyngeal cancer detection based on blood plasma surface-enhanced Raman spectroscopy and multivariate analysis. Biosensors & Bioelectronics. 2010;**25**:2414-2419. DOI: 10.1016/j.bios.2010.03.033

[28] Tan Y, Yan B, Xue L, Li Y, Luo X, Ji P. Surface-enhanced Raman spectroscopy of blood serum based on gold nanoparticles for the diagnosis of the oral squamous cell carcinoma. Lipids in Health and Disease. 2017;**16**:73. DOI: 10.1186/s12944-017-0465-y

[29] Kendall C, Day J, Hutchings J, Smith B, Shepherd N, Barr H, et al. Evaluation of Raman probe for oesophageal cancer diagnostics. Analyst. 2010;**135**:3038-3041. DOI: 10.1039/c0an00536c

[30] Almond LM, Hutchings J, Kendall C, Day JCC, Stevens OAC, Lloyd GR, et al. Assessment of a custom-built Raman spectroscopic probe for diagnosis of early oesophageal neoplasia. Journal of Biomedical Optics. 2012;**17**:81421. DOI: 10.1117/1.JBO.17.8.081421

[31] Wang J, Lin K, Zheng W, Ho KY, Teh M, Yeoh KG, et al. Simultaneous fingerprint and high-wavenumber fiber-optic Raman spectroscopy improves in vivo diagnosis of esophageal squamous cell carcinoma at endoscopy. Scientific Reports. 2015;**5**:12957. DOI: 10.1038/srep12957

[32] Lin K, Zheng W, Lim CM, Huang Z. Real-time in vivo diagnosis of laryngeal carcinoma with rapid fiber-optic Raman spectroscopy. Biomedical Optics Express. 2016;**7**:3705-3715. DOI: 10.1364/BOE.7.003705

[33] Ming LC, Gangodu NR, Loh T, Zheng W, Wang J, Lin K, et al. Real time near-infrared Raman spectroscopy for the diagnosis of nasopharyngeal cancer. Oncotarget. 2017; **8**:49443-49450. DOI: 10.18632/oncotarget.17703

[34] Feng S, Chen R, Lin J, Pan J, Wu Y, Li Y, et al. Gastric cancer detection based on blood plasma surface-enhanced Raman spectroscopy excited by polarized laser light. Biosensors & Bioelectronics. 2011;**26**:3167-3174. DOI: 10.1016/j.bios.2010.12.020

[35] Chen Y, Chen G, Zheng X, He C, Feng S, Chen Y, et al. Discrimination of gastric cancer from normal by serum RNA based on surface-enhanced Raman spectroscopy (SERS) and multivariate analysis. Medical Physics. 2012;**39**:5664-5668. DOI: 10.1118/1.4747269

[36] Lin K, Wang J, Zheng W, Ho KY, Teh M, Yeoh KG, et al. Rapid fiber-optic Raman spectroscopy for real-time in vivo detection of gastric intestinal metaplasia during clinical gastroscopy. Cancer Prevention Research (Philadelphia, PA). 2016;**9**:476-483. DOI: 10.1158/1940-6207.CAPR-15-0213

[37] International Agency for Research on Cancer. GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012. n.d. Available from: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx [Accessed: Aug 24, 2017]

[38] Kong K, Zaabar F, Rakha E, Ellis I, Koloydenko A, Notingher I. Towards intra-operative diagnosis of tumours during breast conserving surgery by selective-sampling Raman micro-spectroscopy. Physics in Medicine and Biology. 2014;**59**:6141-6152. DOI: 10.1088/0031-9155/59/20/6141

[39] Feng S, Huang S, Lin D, Chen G, Xu Y, Li Y, et al. Surface-enhanced Raman spectroscopy of saliva proteins for the noninvasive differentiation of benign and malignant breast tumors. International Journal of Nanomedicine. 2015;**10**:537-547. DOI: 10.2147/IJN.S71811

[40] Depciuch J, Kaznowska E, Zawlik I, Wojnarowska R, Cholewa M, Heraud P, et al. Application of Raman spectroscopy and infrared spectroscopy in the identification of breast cancer. Applied Spectroscopy. 2016;**70**:251-263. DOI: 10.1177/0003702815620127

[41] Canetta E, Mazilu M, De Luca AC, Carruthers AE, Dholakia K, Neilson S, et al. Modulated Raman spectroscopy for enhanced identification of bladder tumor cells in urine samples. Journal of Biomedical Optics. 2011;**16**:37002. DOI: 10.1117/1.3556722

[42] Lin D, Feng S, Pan J, Chen Y, Lin J, Chen G, et al. Colorectal cancer detection by gold nanoparticle based surface-enhanced Raman spectroscopy of blood serum and statistical analysis. Optics Express. 2011;**19**:13565-13577

[43] Gonzalez-Solis JL, Martinez-Espinosa JC, Salgado-Roman JM, Palomares-Anda P. Monitoring of chemotherapy leukemia treatment using Raman spectroscopy and principal component analysis. Lasers in Medical Science. 2014;**29**:1241-1249. DOI: 10.1007/s10103-013-1515-y

[44] Takamori S, Kong K, Varma S, Leach I, Williams HC, Notingher I. Optimization of multimodal spectral imaging for assessment of resection margins during Mohs micrographic surgery for basal cell carcinoma. Biomedical Optics Express. 2015;**6**:98-111. DOI: 10.1364/BOE.6.000098

[45] Zhou X, Chen G-Y, Zhang J-M, Fan C-Z, Liang E-J, Guo Y, et al. Research on early diagnosis of esophageal cancer by Raman spectroscopy of human hemoglobin. Guang Pu Xue Yu Guang Pu Fen Xi. 2013;**33**:2989-2992

[46] Wang W, Pan Z, Tang W, Li Y, Fan C. Research on early diagnosis of gastric cancer by the surface enhanced Raman spectroscopy of human hemoglobin. Guang Pu Xue Yu Guang Pu Fen Xi. 2015;**35**:3402-3405

[47] Pahlow S, Meisel S, Cialla-May D, Weber K, Rösch P, Popp J. Isolation and identification of bacteria by means of Raman spectroscopy. Advanced Drug Delivery Reviews. 2015;**89**:105-120. DOI: 10.1016/j.addr.2015.04.006

[48] Krafft C, Popp J. The many facets of Raman spectroscopy for biomedical analysis. Analytical and Bioanalytical Chemistry. 2015;**407**:699-717. DOI: 10.1007/s00216-014-8311-9

[49] Willemse-Erix DFM, Scholtes-Timmerman MJ, Jachtenberg J-W, van Leeuwen WB, Horst-Kreft D, Bakker Schut TC, et al. Optical fingerprinting in bacterial epidemiology: Raman spectroscopy as a real-time typing method. Journal of Clinical Microbiology. 2009;**47**:652-659. DOI: 10.1128/JCM.01900-08

[50] Willemse-Erix D, Bakker-Schut T, Slagboom-Bax F, Jachtenberg J, Lemmens-den Toom N, Papagiannitsis CC, et al. Rapid typing of extended-spectrum beta-lactamase- and carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* isolates by use of SpectraCell RA. Journal of Clinical Microbiology. 2012;**50**:1370-1375. DOI: 10.1128/JCM.05423-11

[51] Voor In't Holt AF, Wattel AA, Boers SA, Jansen R, Hays JP, Goessens WHF, et al. Detection of healthcare-related extended-Spectrum Beta-lactamase-producing Escherichia Coli transmission events using combined genetic and phenotypic epidemiology. PLoS One 2016;**11**:e0160156. DOI: 10.1371/journal.pone.0160156

[52] Ngo HT, Gandra N, Fales AM, Taylor SM, Vo-Dinh T, Sensitive DNA. Detection and SNP discrimination using ultrabright SERS nanorattles and magnetic beads for malaria diagnostics. Biosensors & Bioelectronics. 2016;**81**:8-14. DOI: 10.1016/j.bios.2016.01.073

[53] Draz MS, Lu X. Development of a loop mediated isothermal amplification (LAMP) – surface enhanced Raman spectroscopy (SERS) assay for the detection of *Salmonella enterica* serotype Enteritidis. Theranostics. 2016;**6**:522-532. DOI: 10.7150/thno.14391

[54] Harz M, Kiehntopf M, Stockel S, Rosch P, Straube E, Deufel T, et al. Direct analysis of clinical relevant single bacterial cells from cerebrospinal fluid during bacterial meningitis by means of micro-Raman spectroscopy. Journal of Biophotonics. 2009;**2**:70-80. DOI: 10.1002/jbio.200810068

[55] Dekter HE, Orelio CC, Morsink MC, Tektas S, Vis B, Te Witt R, et al. Antimicrobial susceptibility testing of gram-positive and -negative bacterial isolates directly from spiked blood culture media with Raman spectroscopy. European Journal of Clinical Microbiology & Infectious Diseases. 2017;**36**:81-89. DOI: 10.1007/s10096-016-2773-y

[56] Driskell JD, Zhu Y, Kirkwood CD, Zhao Y, Dluhy RA, Tripp RA. Rapid and sensitive detection of rotavirus molecular signatures using surface enhanced Raman spectroscopy. PLoS One. 2010;**5**:e10222. DOI: 10.1371/journal.pone.0010222

[57] Zhan L, Zhen SJ, Wan XY, Gao PF, Huang CZ. A sensitive surface-enhanced Raman scattering enzyme-catalyzed immunoassay of respiratory syncytial virus. Talanta. 2016;**148**:308-312. DOI: 10.1016/j.talanta.2015.10.081

[58] Brookmeyer R, Evans DA, Hebert L, Langa KM, Heeringa SG, Plassman BL, et al. National estimates of the prevalence of Alzheimer's disease in the United States. Alzheimer's Dement. 2011;**7**:61-73. DOI: 10.1016/J.JALZ.2010.11.007

[59] Ryzhikova E, Kazakov O, Halamkova L, Celmins D, Malone P, Molho E, et al. Raman spectroscopy of blood serum for Alzheimer's disease diagnostics: Specificity relative to other types of dementia. Journal of Biophotonics. 2015;**8**:584-596