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Optical Spectroscopy and Computational Methods in Biology and Medicine

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Preface

This book is focus mainly on vibrational spectroscopy, Raman scattering and infrared absorption, and their applications in biology and medicine. When talking about new techniques, beside imaging or surface-enhanced spectroscopy also the investigation of optical activity of the samples became significantly common. In view of the fact that beside vibrational spectroscopy also electronic absorption is presented here as a potent and complementary tool to study absolute configuration, the overall topic of this book is named "optical spectroscopy", however the main part is devoted to vibrational methods.

Since the sample investigation is often complicated, especially when a specific analyte is spread in the biomatrix and occurs in low concentration, it is necessary to support the analysis by theoretical methods including chemometrics or 2D correlation spectroscopy. Demonstrated here approach connecting optical spectroscopy with computer methods is shown as a powerful tool that can be successfully used in biology and medicine to study very complex samples.

The book contains a short introduction followed by a set of examples presenting results obtained by using spectroscopy combined with calculations. New techniques and its special application are based on Surface-Enhanced Raman Spectroscopy (SERS), Raman Optical Activity (ROA), Vibrational Circular Dichroism (VCD), Electronic Circular Dichroism (ECD) and matrix isolation spectroscopy. For biological applications the analysis of secondary and primary plant components in the tissue and single cells is demonstrated. After that, biomedical applications of optical spectroscopy related to disease diagnosis including the identification of illnesses biomarkers. A broad qualitative, quantitative and structural study is possible when the advanced computer methods are used.

Malgorzata Baranska

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Part I
Introduction

Chapter 1
General Overview on Vibrational Spectroscopy Applied in Biology and Medicine

Malgorzata Baranska, Maciej Roman and Katarzyna Majzner

Abstract This chapter contains a short introduction to vibrational spectroscopy followed by an overview on its biological and biomedical applications. The spectroscopic techniques discussed in the book and their special advantages are briefly listed, i.e. Surface-Enhanced Raman Spectroscopy (SERS), Raman Optical Activity (ROA), Vibrational Circular Dichroism (VCD), Electronic Circular Dichroism (ECD) and matrix isolation.

The potential of vibrational spectroscopy is demonstrated by the current state of the art in secondary and primary plant components analysis performed in the tissue and from the single cells. Both Raman and IR spectroscopy are shown as powerful tools in medical diagnosis, cytology and histopathology. A brief overview on biomedical vibrational spectroscopy used to investigate lifestyle diseases is provided.

Keywords Surface-Enhanced Raman Spectroscopy (SERS) • Raman Optical Activity (ROA) • Vibrational Circular Dichroism (VCD) • Electronic Circular Dichroism (ECD) • Matrix isolation • Biological and biomedical application

1.1 Vibrational Spectroscopy Methods

Infrared and Raman spectroscopy are among the most widely used techniques in natural sciences. This is due to the fact that vibrational spectroscopy can be applied not only for samples in milligram quantities but microsampling and trace analysis is nowadays done routinely, and measurements take from minutes to fractions of seconds.

The selection rules, which formally constrains the possible transitions of a molecular system, are different for IR and Raman spectroscopy. The classical theory

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has lead to the following criteria: a given normal vibration of a molecule may appear in the IR spectrum if at least one component of the electric dipole moment of the molecule changes during the vibration whereas respectively for the Raman spectrum: at least one component of the polarizability tensor changes. However according to quantum theory, oscillation of a permanent or induced dipole does not produce any radiation. For radiation to be emitted or absorbed, a transition should occur between different vibrational energy levels of the molecule which may be concomitant with loss or gain of energy in the form of electromagnetic radiations. The classical analogue of excitation of a molecule to a higher energy level is vibration with increased amplitude [1].

Also a spatial resolution of both vibrational methods is different. According to the Rayleigh criterion, which defines the resolution obtained from the diffraction limited grating spectrometers and also by a Fourier transform spectrometer that uses triangular apodization, IR spectroscopy is at the limit of 10–12 microns whereas for Raman scattering it is 0.3 microns (for the excitation of 488 nm). Various sampling technique can improve a spatial resolution, however their employment is also limited.

IR and Raman spectroscopy are complimentary methods. Among 3N-6 vibrational modes (N—the number of atoms in a molecule), for some specific functional groups a characteristic frequency can be used for their identification, quantification or conformational study.

Both methods in practice are used as individual techniques, sometimes restricted only to special applications but also providing unusual possibilities and advantages. For the purpose of this book the results of Raman and IR imaging, Surface-Enhanced Raman Spectroscopy (SERS), Raman Optical Activity (ROA), Vibrational Circular Dichroism (VCD), and matrix isolation spectroscopy are presented. Additionally to dichroism and optical activity in vibrational spectroscopy, Electronic Circular Dichroism (ECD) is introduced.

Vibrational spectroscopic imaging greatly extend the possibilities of the conventional IR and Raman spectroscopy. Spectral data can be represented as a picture, presenting chemical information simultaneously from thousands of pixels. The pixel brightness or colour can be derived from any number of spectral parameters, ranging from simple vibrational peak intensities to multivariate parameters. The ability to explore spectral information in a spatially resolve manner can give significant insight into organisational motifs and aid the prediction of molecular functional behaviour. Hopefully the usefulness of an integrated spectral and spatial techniques will result in their application to routine analysis, with a special focus on biological samples and medical diagnosis [1].

Surface Enhanced Raman Spectroscopy (SERS) is a useful analytical technique employed for the identification accompanied by investigation of the most probable orientation of molecules adsorbed on the metal surface [2]. Although SERS spectroscopy is widely applied, the mechanism leading to the surface enhancement is not completely understood yet. Three possible contributions to the enhancement factor have been identified: (i) the surface plasmons resonance in the metal nanoparticles, (ii) a charge-transfer resonance involving shift of electrons between

the molecule and the conduction band of the metal, and (iii) resonances within the molecule itself [3].

VCD, ROA and ECD are applied to study enantiomers, but beside small molecules also large and complex biopolymers e.g. proteins and DNA can be analysed. With the help of *ab initio* calculations the identification of absolute configuration of the molecule is possible. The measurements requires the use of left- and right- circularly polarized light, the spectrometers must be very sensitive, and usually the spectra require a long accumulation. For most biomolecules, i.e. proteins and nucleic acids, the difference in absorbance (scattering) between the two configurations is a few orders of magnitude smaller than the corresponding unpolarized absorbance (scattering).

Most of well known advantages of matrix isolation spectroscopy are related to studies of short-lived molecules and free radicals, but also should be listed: the elimination of hot transitions from the thermally populated vibrational states, sharpness and significantly reduced overlap of the spectral bands, the possibility to study thermally unstable species. In the gas phase, the molecules can be observed unhindered by the environment, and very high-resolution spectra can be used to determine all of the physical properties of a molecular system [1].

1.2 Biological Application

Both IR and Raman spectroscopy can provide important information about the composition and complexity of biological samples. First biological applications of IR spectroscopy were limited to dried plant material and related products because of strong dipole moment of water. The development of Fourier transform (FT) methods pushed the usage of both spectroscopic techniques dramatically. Also better signal-to-noise ratio and shorter time of spectrum acquisition resulted in new applications of these methods. In particular, ATR techniques have improved rapid IR measurements of most liquids such as edible oils, essential oils and solvent extracts of various plant tissues. High accuracy of IR measurements gives an alternative to the conventional methods such as titration and, as a result, IR is most commonly applied for the analysis of dried or non-aqueous plant materials. On the other hand, water has weak Raman scattering properties and consequently Raman methods are more suitable for *in situ* studies of fresh plant materials. Contrary to IR, Raman spectroscopy does not require special sample preparation and optical transparency. Samples can be analyzed directly wet or dry and in many cases nondestructively. Although Raman scattering is weaker than IR absorption, in some cases samples containing lower analyte concentration can be investigated. In particular, two techniques, i.e. resonance Raman and SERS, result in a significant sensitivity enhancement.

Since each functional group in a molecule contributes more or less to the spectral output, vibrational spectra of plant material are usually very complex. The accurate results from spectroscopic analysis can be difficult because of the fact that overlapping and mixing of various vibrational modes occur. Consequently, for many years IR spectroscopy in the middle spectral range (MIR) was used in agricultural studies

only as a qualitative technique for identification and verification of unknown pure substances isolated from extracts or distillates [4]. Nowadays, due to better equipment and modern techniques, FT-IR spectroscopy has become a powerful tool for elucidating the structure, physical properties, and interaction of various plant components. Raman spectra support IR analysis however due to weak scattering some compounds can be difficult to identify.

Vibrational spectroscopy methods can be applied for investigation of primary and secondary plant metabolites. Primary metabolites are defined as those plant constituents, which are essential for the life of plants. It means that they are directly involved in plants normal growth, development, and reproduction. The most important representatives of this group are proteins, lipids, and carbohydrates.

Contrary to primary metabolites, secondary plant metabolites are usually unique to individual plant species and occur in low concentrations. Furthermore, they are not essential to plant cell survival and their importance is related to ecological aspects such as defense against predators, parasites and diseases, interspecies competition, and reproductive processes (colors, smells, etc.). Among a great number of secondary metabolites, the most common are phenolic compounds, terpenoids, alkaloids, polyacetylenes, nitrile compounds, iridoids and chlorophylls. Although their very low concentration in plant material, resonance Raman and FT-Raman spectroscopies provide good spectra with no or little fluorescence [5, 6]. For instance, fruit ripening of various species can be followed by Raman spectroscopy due to the fact that some carotenoids decline when other are accumulated during the ripening process.

Apart from identification of primary and secondary plant metabolites, vibrational spectroscopy gives an opportunity to follow distribution of the metabolites and other plant components simultaneously and directly in plant material (*in situ*). Such distribution studies can be performed using Raman mapping and IR imaging. By combining these techniques with microscopy, molecular information can be obtained with high spatial resolution. There are numerous examples of the usage of *in situ* Raman mapping technique for investigation of plant metabolites distribution in cells, tissues, and whole parts of plants. One of the first Raman mapping studies in plant research was performed on flax stem tissue gaining information on major components (cellulose, lignin, polysaccharides) in different tissue types [7]. The potential of Raman mapping combined with confocal microscope has been shown for the components of cell walls of wood with a high spatial resolution (below 1 μm) [8]. Furthermore, application of polarized laser light provided an insight into changes of orientation of the cell wall constituents [9].

High spatial resolution of Raman microscopy enables characterization of individual compounds in single plant cells. For instance, lipid droplets in xylem [10] and spherical storage compounds in parenchyma cells [8] have been detected in woody plants. Raman spectroscopy can be also applied for distribution studies in single plant cells when imaging techniques are used. Moreover, a single carotenoid crystal has been detected directly in a carrot cell [11]. The cellular distribution of carotenoids with the focus on the individual compounds (β-carotene or astaxanthin) in single cells of various algae was performed [12, 13].

Fast development of FT-IR techniques enabled extensive application of IR imaging for distribution studies of main components in the plant material. Several works have been focused on changes in wood tissues including cell wall modification during maturation [14], fungi degradation [15], steam treatment [16], and transgenic modification [17] to show tissue specific accumulation/degradation patterns. IR imaging has been applied for the analysis of wheat endosperm cell wall composition changes under the impact of environmental conditions [18]. Differentiation between wheat cultivars based on variety of endosperm cell walls [19] was demonstrated. IR imaging technique can be more successful than conventional chemical methods in indicating differences between similar chemotypes, e.g. between wild and mutant type of Arabidopsis [20].

High spatial resolution of IR imaging can be achieved by using a synchrotron source. Synchrotron radiation-based (SR) FT-IR imaging has already been successfully used to examine plant tissue in, e.g. cereals [21]. However, application of FPA detector in IR microspectroscopy has been shown to achieve similar results to synchrotron measurements at the single cell level [22]. Finally, IR imaging based on FPA detector can be applied for bigger areas of a plant such as whole root sections and leaves to observe tissue specific distribution of the studied compounds with comparable spatial resolution and signal-to-noise ratio.

Results from Raman mapping or IR imaging are usually analyzed by comparing integral intensity or by chemometric methods such as Principal Component Analysis (PCA) and Cluster Analysis (CA) to obtain two-dimensional maps and images.

Apart from qualitative analysis of plant material, vibrational spectroscopy is a powerful tool for quantitative studies of individual plant components. This method does not require extraction or separation of the constituents in contrary to widely used chromatographic methods. Vibrational methods allow the quantification of new samples directly from spectral data. Thus, IR and Raman spectroscopy can be used as fast and nondestructive calibration methods for quantification of relevant component contents in plants and related products. Linear calibration based on vibrational spectra and chromatographic data can be used for reliable prediction of the product content as well as for the efficient selection of high-quality single products in industry. It seems to be a powerful tool for a rapid and low-cost alternative quality control method of food production processes.

1.3 Biomedical Application

In the biomedical field it is very desirable to develop innovative and widely used techniques to study the uptake and distribution of bioactive substances. The optical methods do not require any additional labeling or special photophysical properties of the investigated sample. The conventional imaging techniques are often limited by the insufficient sensitivity, specificity and spatial resolution, however the vibrational spectroscopy can be applied to study even single animal cells and various tissues.

Vibrational spectroscopy combined with microscopy has become a powerful diagnostic tool in the biomedical applications. Raman and infrared absorption

spectroscopy appear to be an innovative, powerful, sensitive and non-invasive methods to study the processes taking place inside the cells. IR spectroscopy monitors the level of the main components e.g. proteins, lipids, DNA/RNA, whereas Raman spectroscopy is useful for the assessment of the overall molecular constitution of biological samples, including proteins, nucleic acids, lipids, carbohydrates, heme, carotenoids and inorganic crystals.

The beginnings of biomedical applications of vibrational spectroscopy goes back to the first half and turn of the twentieth century [23–27]. In 1949 Elkan Blout et al. have published one of the first paper where the first spectroscopic experiments on microtomed tissue sections using infrared spectroscopy to study tissue were presented [23]. A normal tissues, neoplastic tissues and important constituents of tissues have been empirically investigated using IR spectroscopy also over 60 years ago by Donald L. Woenley [25]. Recent technological developments have spurred increased interest in Raman microspectroscopy as a sophisticated analytical tool for biomedical applications. The literature repeatedly emphasized the potential impact and importance of these techniques in the application of modern scientific research in the field of tissue engineering and research on new drugs.

The ability to detect diseases or dysfunctions rapidly, non-invasively and unequivocally has multiple benefits. Fast and powerful methods of diagnosis in the initial state of disease allow for early intervention of therapeutic strategies and significant reduction in mortality and morbidity. Detection, identification and tracking of characteristic biochemical markers of disease could be used to monitor the progression of therapy.

Current biomedical studies with application of vibrational spectroscopy are focused on biomedical samples obtained from patients or laboratory animals during surgery, biopsy or *postmortem*, and on cultured cells as well. In recent years there has been a significant increasing interest in the *in vitro* analysis of cells and extracellular matrix components in tissues and *ex vivo* analysis of animal and human tissues by spectroscopic methods for diagnostic purposes. Vibrational spectroscopy opens completely new possibilities for monitoring the content of many chemicals in cells or tissues at the same time at high level of selectivity and resolution. The desire of scientists to have an insight into the biochemical composition of a single cell in an easier, faster and, most importantly, without the labeling in contrast to staining methods, has initiated a number of studies with the use of spectroscopic techniques.

Raman microspectroscopy is able to detect small biochemical changes and their distributions at sub-cellular level [28]. The possibilities to study samples in the aqueous environment allows the investigation of living cells and tracking of changes in their interior under the action of various factors, e.g. monitoring the uptake of drugs, nanoparticles and bioactive compunds, as well as non-chemical factors [29–32].

Many disease syndromes begin at subcellular level, and actually only a few currently used non-invasive techniques allow the study of selected sub-cellular structures in a selective, sensitive and free-labels way. Raman measurements with a spatial resolution ca. ~300 nm allow detection of such small structures as nucleolus, nucleoli, mitochondria, lipid droplets or introduced nanoparticles [33–36]. With the use of confocal Raman spectrometer it is possible to perform three-dimensional imaging

including the position, size and shape of the selected organelles [37]. Changes occurring during the cell cycle, cell death, drug–cell interactions, proliferation differentiation [38–40], or the interaction of cells with various chemicals and materials, can be measured at the biochemical level with high spatial and time resolution.

In the recent years biomedical vibrational spectroscopy has been used to investigate lifestyle diseases, called also diseases of civilization. Lifestyle diseases can include Alzheimer's disease, atherosclerosis, asthma, cancer, chronic liver disease or cirrhosis, type 2 diabetes, heart diseases, metabolic syndrome and many others. Appropriate quantitative approach using Raman or/and infrared spectroscopy combined with chemometric analysis allows to estimate degree of the disease progression.

One direction of the research is focused on the origin, development, treatment, and prevention of cancer—both at the tissue and cellular level. Cancer is one of the most deadly diseases, which modern medicine does not fully understand and is not able to heal. Early detection of cancer through screening based on imaging is probably the major contributor of reduction in mortality for certain cancers. Future developments using Raman spectroscopy and nanoparticles targeted to tumor biomarkers are promising [41]. Raman spectroscopy combined with confocal microscopy can be used for early detection of cancer [42–45] or the analysis of the tumor morphology [46, 47]. Raman and infrared spectroscopy seems to be a promising tool in the diagnosis of bladder [48, 49], prostate [50–52], stomach [53], larynx [54], tonsil [55], lung [56, 57], breast [58–62] and esophagus [63] cancers, as well as basal cell carcinoma [64–65].

Since the first measurements of living cells were achieved by Puppels et al. [66], Raman microspectroscopy was used as a non-invasive and non-destructive tool for probing single living cancer cell while preserving cell integrity and functions, such as adhesion and proliferating capacities. Application of Raman spectroscopy to cell-drug interaction allows for better and faster exploring of disease mechanisms, dependencies and seeking a cure for cancer.

Alzheimer's disease (AD) is a terminal form of dementia resulting from progressive degeneration of the neurons. So far its causes are unknown. Some papers suggest that application of FT-IR spectroscopy could be used in the pathological diagnosis of AD for the classifcation of AD brain tissue (e.g. grey matter), neurotic plaques in the brains, structural characterization β-sheet structure in amyloid fibrils, tau protein and AD-paired helical fragments [67]. Nevertheless because of the penetration depth of infrared light in the tissue (approx. 10 μm) infrared absorption spectroscopy cannot be used for non-invasive diagnosis of AD *in vivo*. For the diagnosis and monitoring of Alzheimer's disease near-IR Raman spectroscopy is a potentially attractive technique because in this system light can penetrate quite deeply into tissue. Mizuno et al. [68], reported a non-destructive examination of human brain tissues and several kinds of brain tumors using near-infrared excited Fourier transform (NIR-FT) Raman spectroscopy. NIR-FT Raman spectra of AD brain tissue show distinct differences from normal tissue spectra that can be used to distinguish AD from normal brain.

A leading cause of death affecting almost one third of humans in developed countries is atherosclerosis. Atherosclerosis is a chronic disease involving degenerative-

productive changes in the intima and central arteries, mainly in the aorta, coronary arteries and cerebral arteries, rarely in the extremities, which leads to a reduction in the light of the arteries and reduce their elasticity by the local deposits of cholesterol, its esters and other lipids [69]. At present, most of the diagnostic techniques commonly used to study patients with atherosclerotic vascular disease (e.g. angiography, nuclear magnetic resonance imaging, electron beam computed tomography or intravascular ultrasound) do not assess the biochemical composition of the vessel wall. Some studies have revealed that Raman and infrared spectroscopy, compared to histopathological analysis as a gold standard, is capable of identifying and classifying the different types of tissues found in the atherosclerotic process of artery *postmortem* [70]. The main purpose of studies with vibrational spectroscopic technique is ability of using label-free optical microscopy to characterize, and thus enable quantitative analyses of different atherosclerotic lesion types. Imaging and quantitative analysis of atherosclerotic lesions was possible by using Coherent Anti-Stokes Raman Scattering (CARS) nonlinear optical microscopy [71]. One of the first real-time investigation utilizing Raman spectroscopy to examine human atherosclerosis *in vivo*, (during femoral bypass procedures) and also to demonstrate the sensitivity of this technique to identify spectroscopic features associated with plaque vulnerability was presented by Motz et al. [72]. Because in vivo Raman spectroscopy does not require the removal of tissue, its success might open several new avenues of research.

Diabetes mellitus (DM) is an increasingly common metabolic disorder triggered by the absence or deficiency of insulin, insulin resistance or by defect in insulin secretion and/or insulin action. As a result hyperglycaemia and profound perturbations in carbohydrate, fat and protein metabolism are observed. These biochemical alterations of cellular metabolism in diabetes include changes in biochemical profile of vascular wall as has been already detected by FT-IR spectroscopy. Variations in spectral parameters can be used in early diagnosis of DM, which may also stimulate the development of patient monitoring devices in future, what can be important in early detection of metabolic disorders. There are only a few reports of vibrational spectroscopy focused on diabetic samples [73] and measurements of glucose concentrations in blood [74]. FT-IR spectroscopy is very informative to differentiate diabetic tissues from healthy ones at the molecular level [75].

Raman spectroscopy is a valuable tool to investigate and follow an oxidative stress, which is one of the potential marker of lifestyle diseases. Oxidative stress is associated with increased production of reactive oxygen species (ROS) and impaired antioxidant mechanisms. Direct observation of reactive oxygen species (ROS) and oxidative stress using vibrational spectroscopy is not possible, but information about ROS can be obtained through the analysis of the effects of ROS on cells and tissues. Krafft et al. [36] presented a novel description of stress-induced changes at subcellular level (nucleus, cytoplasm, vesicles, inclusion bodies, and the peripheral membranes) and apoptosis of cells based on Raman microspectroscopy imaging. Raman spectroscopy has been shown to be also useful in determining correlation between carotenoid antioxidants in living human tissues and risk for malignancies or other diseases associated with oxidative stress, such as e.g. cancer,

atherosclerosis or Alzheimer's disease [76, 77]. Development of the methodology should allow for the diagnostic assessment of biochemical changes caused by the oxidative stress.

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Chapter 2
Integrating Optical Spectroscopy and Chemometric Methods

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Abstract In this Chapter, we describe the usage of several chemometric and numerical techniques to analyse of UV-Vis sets of spectra. The fundamentals of each technique are briefly presented with examples of its applications. This approach allows obtaining deeper insight in studied system. These methods can be used not only to analyze aggregation process, as it was presented in the Chapter, but also to study the interaction between small ligands and macromolecules, such as DNA. Determination of the number of formed complexes and the binding constant of interaction ligand/macromolecules can be received with this methodology.

Keywords Chememetrics • UV-Vis spectra • Aggregation • Imidazoacridines

2.1 Introduction

In chemistry, we have been often dealing with a set of measured data, which are in fact a mixture of the information and the noise. In many cases, the magnitude of the noise is as much great as the information that significantly hinders the ability to find the interesting results in multidimensional data sets [1]. The solutions of such problems are proposed by chemometrics. By means of chemometric techniques we are able to: (i) delete as much noise as possible from the data sets; and, (ii) extract as much information as possible from the multidimensional data [1].

However, to obtain reliable results of chemometrics analysis some special rules should be applied.

The chemical/analytical problem that one would like to solve, has to be defined precisely before starting the experiment. Problems solved by means of chemometric methods can be grouped into four main families: making the

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visualisation of multivariate data set; searching the relationships between sets of data; recognition of internal data structure and making the classification [2]. Each of them needs adequate type of data sets and usage the appropriate chemometric approaches.

The next step is to design carefully the experiment and then perform measurements. The choice of the experimental design depends on the type of information required to verify the research hypothesis, which in fact is the solution of the defined problem. It is indicated to perform as little trials as possible, but selected samples should be representative [1, 2]. In such way, we do not only save time and cost of the whole project but also let make to increase significantly the quality of the results.

Collected data should undergo multi-aspect control procedures to: (i) identify the outliers; (ii) determine of the variable distribution that in some cases indicate the necessity to perform data transformation; and, (iii) determine of interdependences between variables. The main goal of this procedure is to eliminate errors and shorten the time necessary to get reliable results [2].

Only data prepared according to described above procedures can be analysed by means of chemometric techniques. The main approaches of chemometrics data analysis are presented in Fig. 2.1.

Summing up, **chemometrics is the field of science dealing with extracting valuable information from multidimensional data sets by means of mathematical and statistical methods**. However, appropriate usage of chemometrics requires thinking about its application in each step of experiment: from the problem defining to the analysis of obtained results.

2.2 Chemometrics and Numerical Approach to a Set of Spectra

One of a typical problem in absorption spectroscopy is to determine an influence of experimental conditions, such as temperature, ionic strength of the medium or pH, on the electronic spectrum of tested compounds. The experimental condition may affect intensity as well as shape of the spectrum. To detect such changes a set of spectra registered at different experimental condition should be analysed.

From chemometrics point of view each spectrum could be treated as a one-dimensional matrix. Absorptions at different wavelengths are elements of this matrix. A set of spectra registered at different condition forms two-dimensional matrix **X** [3, 4].

2.2.1 Preprocessing of Spectra

Registered sets of spectra, transformed to molar extinction scale according to the Lambert-Beer Eq. (2.1) have to be preprocessed.

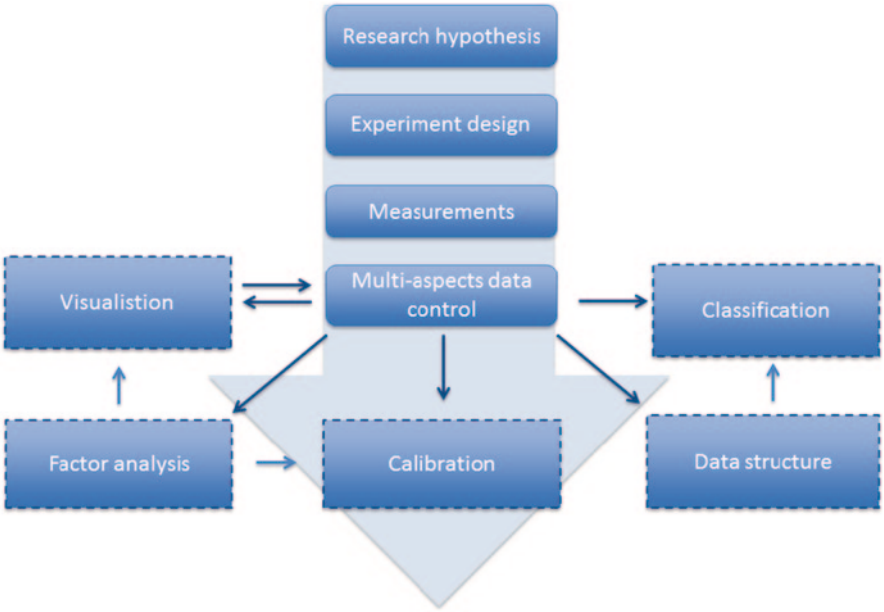

Fig. 2.1 The overview of chemometrics methods

$$A_\lambda = \varepsilon_\lambda cl \tag{2.1}$$

A_λ absorbance at a given wavelength, expressed in nanometers,
ε_λ the molar extinction coefficient at a given wavelength,
c molar concentration of compound,
l optical path length expressed in centimeters

The first steps of these operations include: baseline correction and dilution correction. Several techniques applied to perform baseline correction are summarized by Kohler et al. [5].

Obtain, corrected and transformed results are organize into matrix **X**, presented in Table 2.1 consists n rows (wavelengths, λ) and m columns (spectra obtained for the m increasing concentrations of the compound or spectra registered at different temperatures, pH, etc.). Each element of this matrix corresponds to extinction values calculated for each sample in following wavelengths.

For each column of matrix, the mean values and standard deviation values can be calculated for whole range of wavelengths or for selected range of wavelength, for example range characteristic for research chromophore. These two parameters can be used for measuring changes in intensity of the spectra [3, 4]. To minimalize influence of changes in intensity of spectra, and in this way study only changes into its shapes, sets of spectra should be transformed. The most popular transformations are centring and autoscaling.

Table 2.1 Matrix X obtain as a result of 1st step preprocessing operations of set of UV-Vis spectra

Wavelength	Sample 1	Sample 2	…	Sample m
λ_1	$\varepsilon_{1,1}$	$\varepsilon_{1,2}$	…	$\varepsilon_{1,m}$
λ_2	$\varepsilon_{2,1}$	$\varepsilon_{2,2}$	…	$\varepsilon_{2,m}$
.	.	.	…	.
λ_n	$\varepsilon_{n,1}$	$\varepsilon_{n,2}$	…	$\varepsilon_{n,m}$

Centring the data is the linear transformation performs to make all variables coincide with the beginning of the coordinate system. It is done by subtracting from each value x_{ij} of data organized into matrix **X** corresponding mean value of jth variable to obtain new matrix **X′**, according to formula [1]:

$$x'_{ij} = x_{ij} - \mu_j \tag{2.2}$$

The autoscaling is made by diving by centered data by the standard deviation of the jth variables according to formula (2.2). As a result, we obtained the normalized matrix **Z** [2]:

$$z_{ij} = \frac{x_{ij} - \mu_j}{\sigma_j} \tag{2.3}$$

where:

x_{ij} molar extinction coefficient for the i-th wavelength of the j-th spectrum
μ_j the average value of the molar extinction coefficients of the j-th spectrum
σ_j standard deviation of the molar extinction coefficients of the j-th spectrum.

Spectrum after standardization can be treated as a vector of unit length. A set of spectra creates a bunch of vectors which have common origin in the k-dimensional hyperspace, where k is the number of spectral forms present in the analyzed samples. A bunch of vectors for recorded spectra is limited by vectors representing the spectra of pure ingredients. An example for two component mixture (k = 2) is shown in Fig. 2.2.

2.2.2 Internal Order of a Matrix and Number of Variety Sources

The Principal Component Analysis (PCA) [6] is a special example of projection pursuit techniques, in which variance is used as a projection index. PCA is mainly used for modeling, compressing and visualizing multidimensional data [7–10]. Application PCA in the relationship analysis involves two basic tasks: graphical

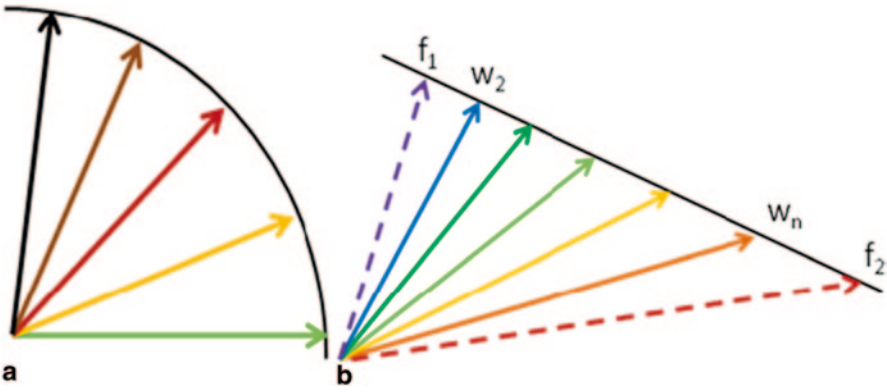

Fig. 2.2 Vectors of the two component mixture in the space of two main components: **a** standardized spectrum. **b** centered spectrum

presentation of the dimensional dependence and reducing the dimensionality of the problem. In this technique, set of correlation coefficient between variables in multivariate space might be transformed to equivalent set of orthogonal factors. The principal components are new orthogonal variables (which are expressed as a linear combination of original variables) and they maximized description of the data variance.

The PCA analysis allows to determine the internal dimension of the matrix of the standardized spectra. This dimension is equal to the number of spectral forms presented in the samples. If the spectra of individual species are not excessively correlated, it is equal to the number of significant principal components of this matrix. Unfortunately, when the spectral forms have very similar spectra, as is usual in the case of aggregation, the analysis of the residual spectra have to be used to determine the number of spectral species in the samples [3, 4].

The principal components and their loadings permit to complete reconstruction of the standardized spectra matrix according to the equation:

$$\mathbf{Z}_{nm} = \mathbf{P}_{nm}\mathbf{L}_{nm} \tag{2.4}$$

where:

$\mathbf{P}_{nm}$ matrix of m principal components,
$\mathbf{L}_{nm}$ matrix of loadings.

If in the spectra reconstruction we use only j first principal components we obtain following relationship:

$$\mathbf{Z}_{nm} = \mathbf{P}_{nj}\mathbf{L}_{jm} + \mathbf{E}^{(j)} \tag{2.5}$$

$\mathbf{E}^{(j)}$ matrix represents the residual spectra of j order. The residual spectra for $j<k$ have the character of the differences spectra, for $j \geq k$ the residual spectrum represents random noise. Thus, further analysis of the residual spectra allows to specify the correct value of k.

2.2.3 Numerical Spectrum Decomposition Technique and Physicochemical Model of a Process

Applying PCA analysis let us know not only the number of spectral forms presented in the samples, but also the first spectra approximation, however, does not allow to designate individual forms, because the principal components are obtained with the assumption of perfect orthogonality.

Therefore, next step in chemometrical analysis of sets of spectra is to obtain the spectra of indywidauals and estimation of its molar fractions. To reach this goal, **Z** matrix should be expressed as a multiplication of the two other matrices: the matrix of components **B**, in which the columns represent the spectrum of pure spectral forms, and matrix **X**, which columns represent different forms of molar fractions [3, 4].

$$\mathbf{Z} = \mathbf{BX} \tag{2.6}$$

Knowing that the ends of the centered vectors of two components mixtures are on a straight line (Fig. 2.2b), the position of the spectra of pure components on this line is unknown. Therefore, in order to solve the Eq. (2.6) there is need to use the iterative procedure called Numerical Spectrum Decomposition [1]. To determine spectra of the complexes, as well as relative amount of each species, an iterative self-consisting procedure—multivariate curve resolution-alternating least squares (MCR-ALS) has to be used [11]. The most outer vectors w1 i wm participate the most within the spectral forms according to the equation.

$$w_1 = x_{11}f_1 + x_{21}f_2 \quad x_{11} \approx 1, x_{21} \approx 0 \tag{2.7}$$

$$w_m = x_{1m}f_1 + x_{2m}f_2 \quad x_{1m} \approx 1, x_{2m} \approx 0 \tag{2.8}$$

In addition, in the two components mixtures, the molar ratios should not be negative, and the sum of the molar fractions of the two forms must be equal to 1:

$$x_{11} + x_{21} = 1 \tag{2.9}$$

$$x_{1m} + x_{2m} = 1 \tag{2.10}$$

Using the Eq. (2.9) and (2.10) molar fraction x_{11} i x_{2m} can be determined:

$$x_{11} = 1 - x_{21} \tag{2.11}$$

$$x_{2m} = 1 - x_{1m} \tag{2.12}$$

After substituting these molar fractions into formulas (2.11) and (2.12) set of two equations were obtained:

$$\begin{cases} w_1 = x_{11} f_1 + (1 - x_{11}) f_2 \\ w_m = (1 - x_{2m}) f_1 + x_{2m} f_2 \end{cases} \tag{2.13}$$

These equations can be solved due to the spectrum of pure form. Assuming further indications:

$$\alpha = \frac{1}{x_{11}} > 1 \tag{2.14}$$

$$\beta = \frac{1}{x_{2m}} > 1 \tag{2.15}$$

the following formulas are obtained:

$$f_1 = \frac{\alpha w_m - (\alpha - 1)\beta w_m}{1 - (\alpha - 1)(\beta - 1)} \tag{2.16}$$

$$f_2 = \frac{\beta w_m - \alpha(\beta - 1) w_1}{1 - (\alpha - 1)(\beta - 1)} \tag{2.17}$$

Coefficients α and β are matched numerically during fitting a physicochemical model of the process studied using the Nelder–Mead simplex method [12, 13]. Obtaining optimal values of α and β is equivalent to determine the spectra of pure species. Determination of the spectra of pure spectral forms allow to calculate the matrix Eq. (2.6) because of the mole fractions. Molar fraction of the individual components in various conditions obtained by this method, could be used to determine the specific parameters of the model used.

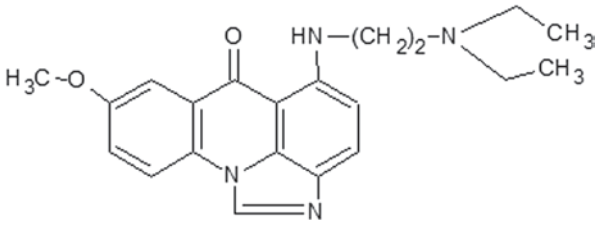

Fig. 2.3 Chemical structure of C-1330

2.3 Influence of Agregation on UV/Vis Spectrum—A Case Study

Acridine belong to a group of polycyclic heteroaromatic compounds and exhibit a broad spectrum of biological activity including antiprotozoal, antibacterial, antiviral and antitumor activity [7, 14]. Imidazoacridinones (IA) derivaties are a group of acridine antitumor drugs synthesized in Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology. The biological activity of the imidazoacridinones has now been extensively investigated. Published data suggested that imidazoacridinone drugs are capable of binding physicochemically to DNA [15, 16]. The nature of these investigation and their relevance to cytotoxic and antitumor properties of IA remains unknown.

Imidazoacrinononones tend to aggregate in diluted solutions, leading to dimer formation, and sometimes even higher order aggregates. Driving forces of this process are hydrophobic interactions. Kinetic information of aggregation process are very helpful to understand molecular interaction such as micelle formation of amphiphilic substances and the binding of small ligands to macromolecules [17–19]. In spite of many studies, the mechanism of compounds self-aggregation seems to be not fully understood because of the experimental conditions, which are different from study to study.

The most useful method to check if some compounds self-aggregate in aqua solution is spectroscopic analysis with increasing drug concentration. The following procedure of results analysis may be used to study the aggregation of various derivatives. An example of such derivative is imidazoacridinon C-1330 (Fig. 2.3), synthesized in Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology [20]. The samples of imidazoacridinon C-1330 were prepare in various concentrations (20 μM to 1 mM) by dilutions. The absorption spectra at different concentration were recorded. Obtained spectra were transformed into the molar scale using Lambert-Beer Eq. (2.1), and then standardized according to Eq. (2.3).

Figure 2.4 represents the set of standardized spectra obtained for C-1330 in different drug concentration. The compound has a strong absorption band in the visible field of the spectrum. Two maximum are observed in the spectra. For the lowest concentration of the compound, they are located at $\lambda = 371$ and $\lambda = 423$ nm. In real spectra, there are two isosbestic points at a wavelength $\lambda = 383$ and $\lambda = 431$ nm.

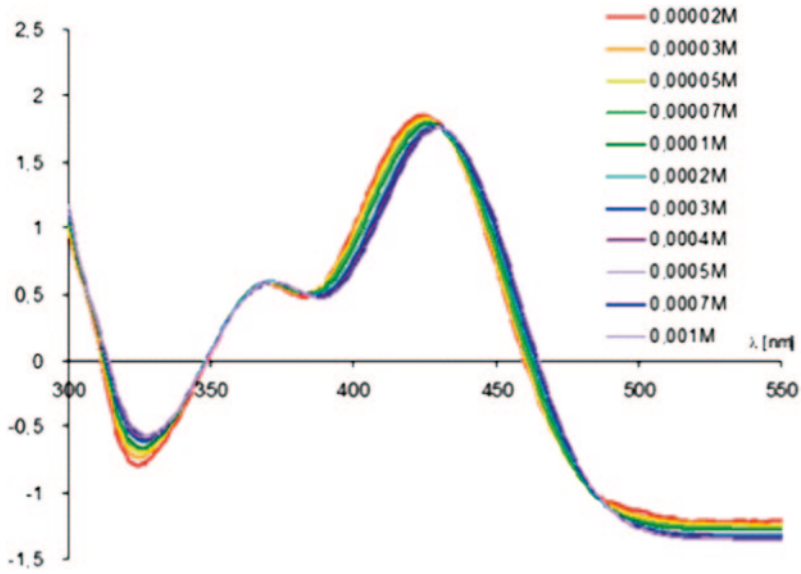

Fig. 2.4 Set of standardized spectra representative for C-1330 in buffer with 5 mM NaCl

Table 2.2 Eigen-values obtained for set of the C-1330 spectra

	No of PC				
	1	2	3	4	5
Eigen-value	9.9827	0.0170	0.0002	0.0001	0.0000
% of variance	99.83	0.16	0.01	0.00	0.00
Cumulative % of variance	99.83	99.99	100.00	100.00	100.00

Numerical analysis of the spectra for C-1330 was started by determining the number of spectral forms present in the solution. Table 2.2 presents the data obtained by using the PCA method. The following values suggest that the first two principal components (explaining 99.99 % of the total variability) are significant.

Similar results were obtained after careful analysis of the residual spectra (Fig. 2.5). The 1st and 2nd order residual spectra are relatively intensive and demonstrate the presence of absorption bands. Experimental noise prevails only in 3rd or higher order residual spectra. This indicates that there are two spectral forms in the solution: monomer and aggregate.

Using the appropriate transformations as it was presented in Chap. 2.3. there were possible to reproduce spectra of pure spectral forms. Figure 2.6 shows such spectra for C-1330. The maximum absorption of the monomer occurs at 423 nm. In the spectra of the aggregate exhibit characteristic batochromic effect with the maximum absorption at the 431 nm. The results of our studies indicate that the presence as well as proportions of particular spectral forms is dependent on the overall concentration of studied compound.

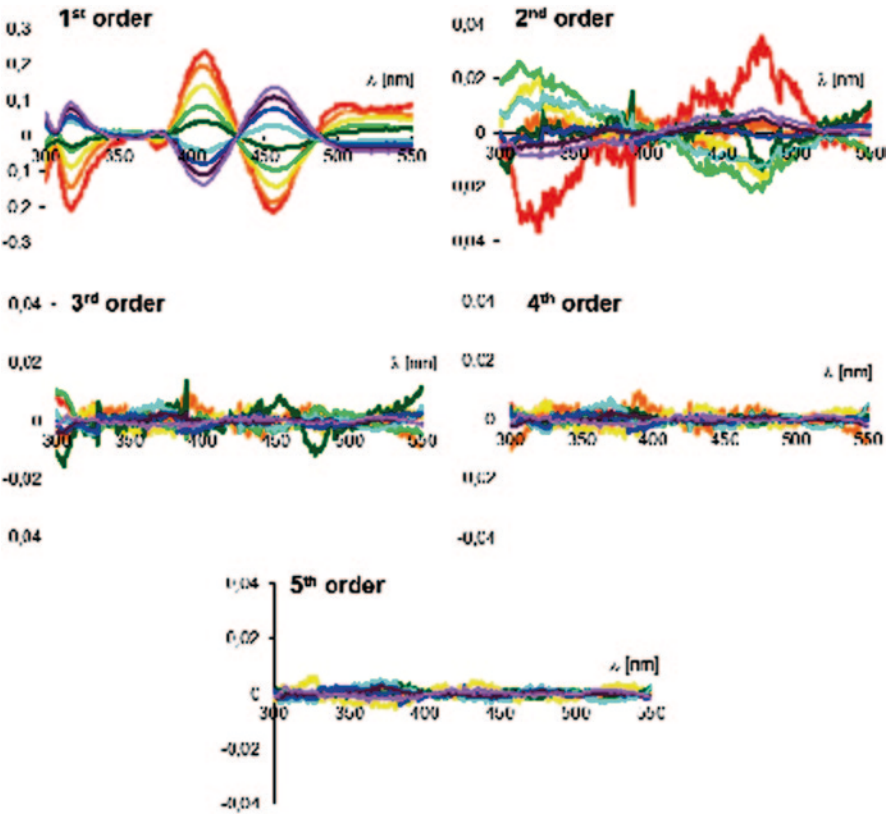

Fig. 2.5 Further residual spectrum obtained for the C-1330 in 5 mM NaCl solution in the PCA method

Obtained molar fraction of the individual components in various concentrations was used to determine the aggregation constant K_A according to the formula:

$$K_{Ai} = \frac{1 - \sqrt{x_{mi}}}{C_{ti} x_{mi}} \tag{2.18}$$

Where

C_{ti} the total concentration of the compound in the i-th solution,
x_{mi} the molar fraction of the monomer calculated from the Eq. (2.6).

The estimation of the m aggregation constant—one for each compound concentrations (for each spectra) were obtained. Than the average value and the standard deviation were calculated: $K_A = (6,327 \pm 0,218) * 10^3 M^{-1}$.

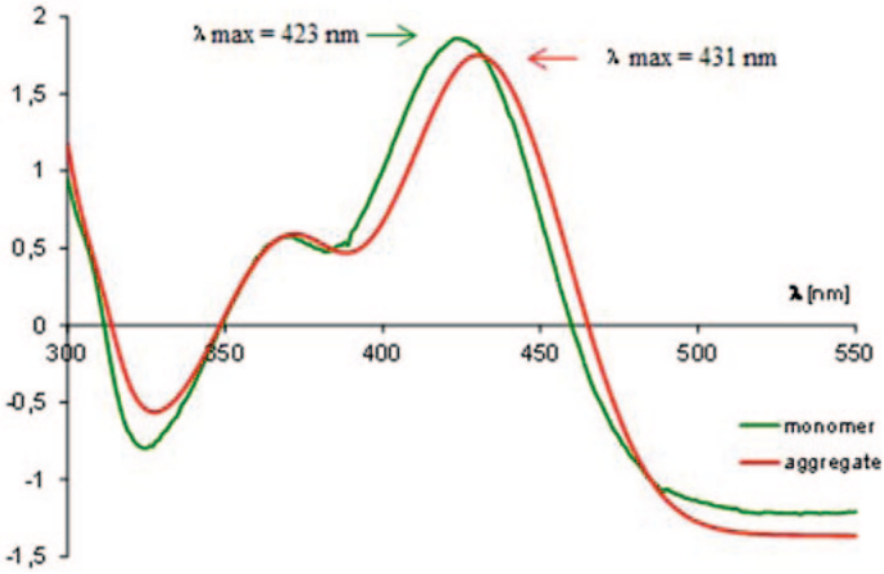

Fig. 2.6 Standardized spectra for monomer (*red line*) and aggregate (*green line*) for C-1330 in 5 mM NaCl

2.4 Conclusions

Application of chemometric and numerical methods can be used to analyse spectroscopic spectra. We presented these applications for the aggregations process that give interesting results even when the spectra of spectral forms presented in the sample differ slightly. The presented approach is universal if the model of the process is known. This methods can be also used to study the interaction between small ligands and macromolecules, such as DNA, for determining the number of formed complexes and the binding constant of interaction ligand/macromolecules. The chemometrics approach to an experimental data allows obtaining deeper insight in studied system. Application of various statistical methods is particular useful when classical simplified assumption is not valid.

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Part II
New Techniques and Special Applications

Chapter 3
Surface-enhanced Raman Scattering (SERS) in Bioscience: A Review of Application

Jolanta Bukowska and Piotr Piotrowski

Abstract This article reviews some recent applications of surface-enhanced Raman scattering (SERS) spectroscopy in biology, biochemistry and biomedicine. We start with a short description of theoretical background of Raman scattering enhancement by the plasmonic nanoparticles. SERS is a phenomenon observed for molecules in a close proximity to the surface of metallic nanostructures. We present an overview of SERS substrates fabricated using various physical and chemical methods. SERS spectroscopy, which combines very high sensitivity with molecular specificity, is a powerful technique for studying biologically important systems, ranging from simple molecules like amino acids, to extremely complex samples such as living cells and tissues. We demonstrate great potential of SERS not only for detection and identification of (bio)molecules, but also in monitoring various biochemical processes. The strategies that are used for biosensing with the aid of SERS spectroscopy are briefly described.

Keywords Surface-enhanced Raman scattering (SERS) • SERS of biological molecules • Biosensors • Intracellular SERS • Nanoparticle probes

3.1 Introduction

Inelastic scattering of light by molecules, utilized by Raman spectroscopy, is nowadays commonly used to provide valuable information on molecular structure as well as chemical composition of the studied samples. Conventional (or normal) Raman spectroscopy suffers from very low scattering cross-sections compared with other spectroscopic methods. This significantly limits its applications to investigate molecules in solutions at low concentrations, which is a common requirement in analytical chemistry and biological systems. The simplest way to increase Raman intensity is exciting the spectrum with the laser beam of the energy corresponding to the energy difference between ground and excited electronic state. This effect, called resonance Raman (RR) scattering allows us to enhance the spectrum several orders of magnitude. However, in case of colorless samples, this method requires excitation in ultraviolet (UV), while the most popular light sources in Raman

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spectroscopy are visible or near infrared lasers. Another, more advanced possibility to increase the Raman scattering intensity is the use of non-linear effects such as stimulated Raman scattering. However, the most effective way of amplification of the Raman scattering is offered by surface enhanced Raman scattering (SERS) spectroscopy. In SERS, Raman signal may be enhanced by as much as 11 orders of magnitude. Typical SERS enhancement factors are of order of 10^4–10^6. Further enhancement of the Raman spectrum is obtained when the laser excitation energy corresponds to electronic transition of the molecule (resonance Raman). In this effect, called surface enhanced resonance Raman scattering (SERRS), enhancement factors can reach 10^{11}, allowing detection at a single molecule level. Combined enhancement processes provide a Raman signal of sensitivity at least equivalent or even higher than that of fluorescence. Very high sensitivity, selectivity and ability to identify molecular species, together with other advantages make SERS/SERRS spectroscopy an exceptionally attractive tool for variety of applications in bioscience. SERS may compete successfully with other spectroscopic methods in bioscience, such as fluorescence.

Giant amplification of the Raman signal in SERS is generated by metallic nanoparticles (typically silver, gold and copper), on which target molecules are adsorbed. The first surface-enhanced Raman spectrum of pyridine adsorbed on a silver electrode was observed by Fleischmann and coworkers in 1974 [1] at the University of Southampton, but in fact they did not recognize the importance of their results. The first explanation of this new phenomenon in terms of a giant surface enhancement of the Raman scattering, observed by Fleischmann et al., was reported by Jeanmaire and van Duyne in 1977 [2]. Since 1977, many papers on both theoretical and practical aspects of SERS spectroscopy have been published. In the last several years, popularity of surface-enhanced Raman spectroscopy has grown rapidly. Among a great variety of applications, these connected with biomolecule detection develop extremely quickly.

3.2 SERS Phenomenon—Background and Mechanism of Enhancement

Phenomena concerning interactions between matter and light might be roughly divided into three cases: absorption and emission of light, scattering—when, as opposed to emission, angle distribution of radiated photons is observed—and reflection, when the wave vector changes its direction at an interface between two media according to the laws of reflection. Raman effect falls into the second category and is defined more accurately as inelastic scattering of light, which means that the energy of the scattered photons differs from the incident ones. Thanks to this energy difference, Raman spectroscopy informs us about energies of rotational and vibrational modes of the studied molecule. Raman spectrum contains a set of bands, which is characteristic for a given molecule, thus identifies the sample, providing its so called chemical fingerprint.

Vibrational Raman effect is classically described by Placzek's theory, which is a simplified depiction of interaction between a molecule and electromagnetic wave that induces dipole moment in the molecule. As far as oscillation Raman effect is concerned, one needs to realize that molecular vibrations influence properties of the charge distribution in the molecule. Therefore, dipole oscillates with frequencies ν_0, $\nu_0 - \nu_{osc}$, and $\nu_0 + \nu_{osc}$, emitting light of frequency equal to its own (ν_0 being frequency of incident light beam and ν_{osc}: frequency of molecular vibrations).

As it follows from the classical description, two types of Raman scattering are distinguished: when the energy of the scattered photons is lower or higher than the energy of the incident ones. It is explained as losing or gaining energy to vibrational or rotational modes of the molecule.

Unfortunately, Raman effect concerns only a small number of photons; vast majority of incident photons is scattered elastically without the energy change. Raman scattering involves only 1 of about 10^7 photons. Such obstacle prevented this technique from effectively developing in application fields for years. It was only in 1970s when Fleischmann et al. [1] observed a sudden rise in the Raman scattering intensity of the molecules adsorbed on a roughened electrode surface. Even though the contribution of the metal surface to the enhancement was not initially recognized, this technique is fully referred to as surface-enhanced Raman scattering (SERS) nowadays. Intrinsically weak Raman scattering can be enhanced thanks to the interaction between all three components of the system: light, adsorbed molecule and the metal surface. Nature of the effect, which is a borderline case between chemistry and physics, makes it necessary to combine languages of both fields in order to fully describe its mechanism. Theories explaining SERS belong to one of the two categories: chemical and electromagnetic ones. It is now well known that the dominant contribution to the enhancement arises from the electromagnetic effect. However, some observations, such as stronger enhancement of the Raman scattering of chemically adsorbed molecules as compared to physically adsorbed ones cause that chemical mechanism should be also taken into account.

Chemical explanation of the surface-enhanced Raman scattering mechanism makes use of charge-transfer theory. It focuses on the influence of the metal surface on energy levels of adsorbed molecules [3]. According to the chemical theory, Raman intensity gain in SERS is obtained by matching the energy of the incident beam with the energy of electron transition between Fermi level of the metal and a molecular level of the adsorbate. Situation when intensity of Raman scattering rises due to laser-induced intermolecular transitions is analogous to resonance Raman mechanism. In that case, enhancement of the Raman scattering is obtained when a laser beam of energy equal to electronic transition of the molecule is applied.

Some features of SERS prove that chemical mechanism plays a role in the enhancement; potential-dependent experiments show that the phenomenon might be tuned to a certain beam energy through applying appropriate bias to the metal adsorbent. However, there are features that cannot be understood on the ground of charge-transfer theory. It turns out that every SERS-active metal has its own energy work range. It is widely known that silver nanostructures yield the best enhancement when exposed to blue/green laser beam; on the other hand, red light supports SERS

on gold structures. In addition, enhancement factor depends on electric permittivity of the medium. This inconsistency led to the second theory of SERS: electromagnetic one. It explores the influence of electromagnetic wave on a roughened surface. First attempt to describe SERS electromagnetically was dipole image theory. It presented a molecule as an oscillating electric dipole. Vibrations of the induced dipole are caused by oscillating electric compound of the electromagnetic wave. As the molecule is situated near the surface, it polarizes the metal. Thus, image dipole in the metal is created. It, in turn, causes extra polarization of the molecule which completes the feedback loop of interaction leading to the enhancement of the electric field near the metal surface. One of the main flaws of this theory is that it does not answer the question why metal nanostructures on the surface are essential to Raman enhancement. More refined theory includes the idea of surface plasmon resonance (SPR). Physicists define plasmons as collective oscillations of electron plasma (collection of valence electrons in the solid) [4]. Plasmons of different energies describe different excitation modes of such oscillations. Special properties of longitudinal charge density waves propagating at a metal/dielectric interface led to distinction of a subgroup of plasmons: surface plasmons [5]. These quasi-particles can be excited only by a transverse electromagnetic wave. What is more, optical properties of the system change drastically when energies of surface plasmons and incident light beam match. In that case, giant rise in electric field intensity near the interface is observed. This phenomenon is called surface plasmon resonance. For light from near UV/VIS/near IR range (usually used to excite Raman scattering), SPR is possible to obtain for plasmons confined in small metallic nanostructures; then we talk about localized surface plasmons (more accurately localized surface plasmon-polaritons, in order to refer to coupling between plasmons and light). When SPR occurs, molecules situated in the vicinity of such a metal nanostructure are exposed to electric field of intensity magnified several orders of magnitude (in comparison to the incident beam). As a result, electromagnetic enhancement factors reach up to 10^{11} (see critical paper on this topic by Ru et al. [6]). Exceptionally high enhancement factors are seen on the edges of the nanostructures. Such places might be called hot-spots (Fig. 3.1). Needless to say, only selected metals enhance Raman scattering effectively in the visible and near infrared range. Coinage metals, silver, gold and copper, face all the extra requirements needed to provide SERS signal. Extensive outlook on the problem of SERS substrates is presented in sect. 3.

One more thing needs to be highlighted. If one is already acquainted with Raman spectroscopy and knows Raman spectra of investigated molecules, it might be tempting to compare them to SERS spectra. However, they may—and usually they do—differ. Due to adsorption onto the metal surface, energies of molecular vibrations change which leads to modification of the Raman shift of certain bands. Furthermore, selection rules that apply to electromagnetic and charge transfer mechanisms of surface enhancement make some vibrational modes selectively enhanced, dependent on the relative contribution of each mechanism to total enhancement [8]. Thus, relative intensities of the bands in the SERS spectra are also altered in comparison to the normal Raman ones. As normal Raman selection rules do not apply in SERS, some bands ascribed to forbidden transitions may appear. Signifi-

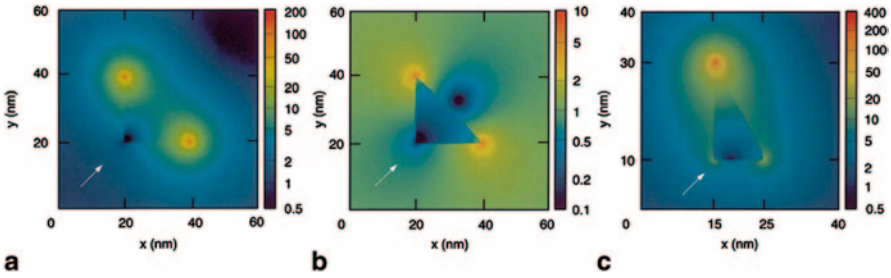

Fig. 3.1 Mathematic modelling of electric field amplitude distribution in the vicinity of different triangular silver nanoparticles as a result of surface plasmon resonance at different excitation wavelengths: (**a**) 412 nm, (**b**) 600 nm, (**c**) 458 nm. (Reproduced from Ref. [7] with kind permission of Elsevier BV)

cant rise in the background is usually observed in the SERS spectra. Among less apparent differences one may recognize: increase of the depolarization ratio of the bands (intensity ratio of the perpendicular to the parallel component of the Raman scattered light) and potential dependence of positions and intensity of the bands for molecules adsorbed on electrodes.

3.3 SERS Supports

For generating SERS spectrum, molecule must be in close contact with SERS-active support. Although many materials have been reported as providing enhancement of the Raman scattering with visible and near-infrared excitation, there are three metals that exhibit the highest enhancement—silver, gold, and copper. Because of high reactivity of Cu, silver and gold are most commonly used as the SERS supports. A key problem in a variety of applications of SERS is obtaining substrates that provide stable, robust, reproducible and effectively enhancing surface. Development of strongly enhancing and highly reproducible substrates is critical in the applications of SERS as an important and efficient spectroscopic technique for studying biomolecules. Some examples of SERS supports are shown in Fig. 3.2.

The main source of the surface enhancement is a very strong electromagnetic field generated by excitation of the localized surface plasmons in the metallic nanoparticles (NP) (see Sect. 2). It is well known that the highest enhancement of the Raman spectrum is obtained when the energy of the laser beam used to excite the spectrum is close to the energy of the surface plasmons. To fulfill the condition of the plasmon resonance for a given excitation laser line, nanoparticles should have proper size, shape and composition. Historically, first substrates used for SERS were electrochemically roughened Ag electrode surfaces [1, 2]. They are still used in some SERS experiments, especially when the applied potential has to be controlled. The advantage of the electrodes is an ease of electrochemical preparation of the rough surface, high enhancement factors and possibility to simply regenerate the surface by subsequent electrochemical dissolution—deposition of the electrode material.

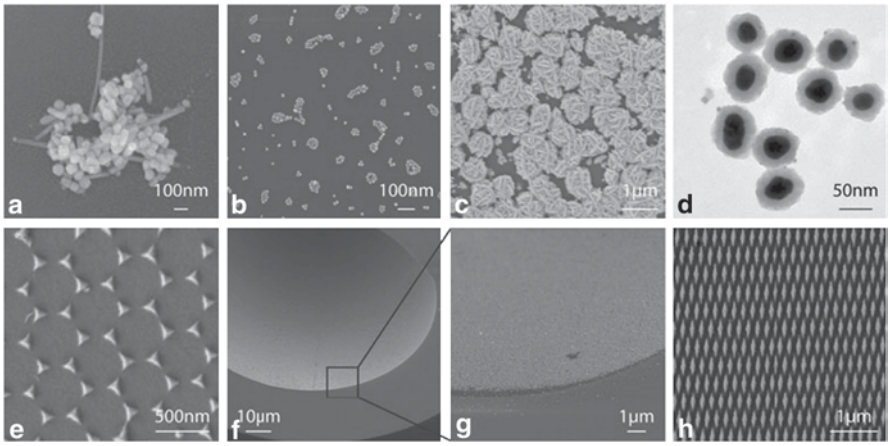

Fig. 3.2 Overview of various SERS substrates: scanning electron microscopy (SEM) (**a**–**c**, **e**–**h**) and transmission electron microscopy (TEM) (**d**) images of **a** aggregated silver nanoparticles produced by a modified Lee–Meisel protocol, **b** gold nanoparticles arranged in clusters, **c** flower-like silver composites prepared by an enzymatic silver deposition, **d** SERS tags for labeling in bioanalytics consisting of gold cores coated by a silica shell, **e** regular arranged metallic triangles by applying nanosphere lithography, **f** optical fibers as templates for SERS substrates, **g** deposited nanoparticles within the optical fiber (magnification of Fig. 3.2f), **h** electron beam lithographic top-down fabrication of periodically arranged gold nanorhombs. (Reproduced from Ref. [33] with kind permission of Springer-Verlag)

Soon after discovery of the SERS effect, metal (Ag and Au) colloids have been introduced as a very effective support (see Fig. 3.2) [9, 10]. Colloidal nanoparticles are produced mainly by chemical reduction of silver or gold cations in solutions, using reducing agents such as sodium citrate, sodium borohydride or hydroxylamine hydrochloride [11]. Metallic nanoparticles in colloidal suspensions are still the most widely used SERS active substrates, being ideal and simply produced substrates for performing measurements in solution. Main drawback of the chemically produced colloidal suspensions is some irreproducibility of the enhancement factors, caused by aggregation processes [12]. To overcome this problem, various methods of fabrication of metal nanoparticles have been developed. For some applications, including those in biology and biomedicine, metal nanoparticles obtained in colloidal suspension are deposited on the solid substrates. However, when colloidal particles isolated on planar substrates were used as the enhancing medium for ultra-sensitive detection of adenine, additional anomalous bands in the background were observed. Significant reduction of the spurious signals was obtained for hydroxylamine-reduced colloid, as compared to the more common citrate- and borohydride-reduced silver colloids [13].

There are many strategies of NP immobilization on solid supports, among which chemical or electrostatic self-assembling are the most popular. Chemical methods consist of functionalization of the solid substrate (usually glass or quartz) with thiol (SH) or amine (NH_2) groups using bifunctional molecules which are able to attach

Ag or Au nanoparticles from the colloid suspension. This strategy was first demonstrated by Chumanov et al. in 1995 [14] and developed by group of Natan et al. [15]. The idea of depositing metal nanoparticles from colloidal suspensions was also successfully applied to the fabrication of three-dimensional (3-D) structures of NP at the solid substrates by repeating chemical binding of the subsequent layers of NP deposit [16–18]. Another way is immobilization of NP by electrostatic attraction of the charged metal particles by oppositely charged solid support. In this strategy the most popular method is covering the solid substrate with positively charged derivatized silanes (e.g. aminopropyltrimethoxysilane) [14] or polymer layer such as poly(diallydimethylammonium chloride), which, as a positively charged polymer, is able to attract the negatively charged colloids [19] or polylysine [20]. Another, less common substrates include surfaces roughened by chemical treatment of solid substrate [21], "island films" deposited on glass and films deposited by evaporation or sputtering substrates [22, 23].

Metallic nanoparticles or other nanostructures can be also fabricated directly on the surface of the solid support using advanced physical methods such as nanolithography [24–28] or micro-contact printing [29]. Thin films of silver or gold can be also vapor-deposited over polystyrene or silica nanospheres of controlled diameter (FON-film over nanospheres), deposited on solid substrate. This method results in highly reproducible and effective supports [30, 31]. Using this technique, van Duyne group developed interesting SERS support by removing the underlying nanosphere mask, thus leaving periodic structure of metal nanoparticles (Fig. 3.2e), deposited on the free space between the nanospheres. As reported [32], supports prepared in this way exhibit very high enhancement factors (of order 10^8).

Plasmonic properties of metal nanoparticles can be significantly changed by tailoring their shape or size. Numerous methods of production of shaped nanoparticles such as nanorods [34, 35] triangles [36], nanocubes [37, 38], polyhedrons [39], stars [40–42] and nanoflowers [43] have been developed. Some of these nanostructures exhibit higher enhancement factors as compared to nanospheres, because of the presence of sharp edges and corners that are able to concentrate extremely strong electromagnetic fields (see Fig. 3.1). Among a great variety of nanoparticles, hollow spheres with differing cavity diameter—which allows tuning the plasmon properties—are very effective enhancers [44].

The possibility of shifting plasmon resonance to the near infrared is sometimes important in biological applications, because of problems with surface photochemistry when using visible radiation (especially on silver) and/or problems with fluorescence background. This red-shift may be also achieved in core-shell nanoparticles built of a spherical silica core of varying dimensions, covered with a thin metal shell [45, 46]. It was confirmed that spherical Au nanoshells, in which plasmon resonance frequencies are controlled by the relative inner and outer radius of the metallic shell layer (see Fig. 3.3), can be used as an effective and reproducible SERS substrate, also for large biomolecules and more complex systems such as DNA and living cells [47–50].

Interesting SERS substrate has been developed by Tian group [52, 53]. They coated Au nanoparticles with a very thin (2–3 nm) silica film that isolates the metal

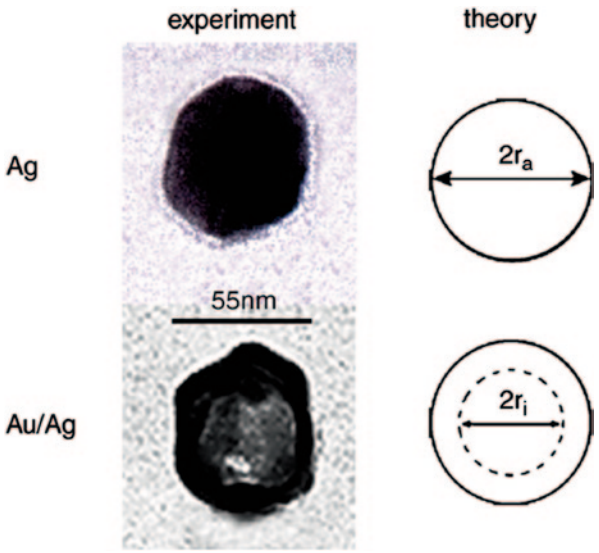

Fig. 3.3 (*Left*) TEM images of a Ag nanoparticle (*top*) and a hollow Au/Ag nanoshell (*bottom*). (*Right*) Schematic diagram of the particle geometry. (Reproduced from Ref. [51] with kind permission of Elsevier Science)

from the environment, preserving high enhancement factor of the shell-isolated nanoparticles (SHINERS—shell-isolated nanoparticle enhanced Raman spectroscopy). Protective layers of silica [54], glass [55] or polyethylene glycol [56] are also applied for metallic nanoparticles labeled with various compounds called Raman reporters. These encapsulated nanoprobes find many applications as SERS probes incorporated in a variety of biological materials (see Sect. 4.2). Larger enhancement of the Raman scattering than that produced by a single nanoparticle (NP) is created at the junction between two nanoparticles in a dimer, in which nanoparticles are separated by nanometer-scale gaps [57–60]. Such NP dimers are sometimes called hot spots. Reproducible fabrication of these nanostructures is however challenging, because the SERS enhancement factor critically depends on interparticle spacing [61].

This short review does not cover all developed SERS supports. Usually, SERS substrates are prepared in a given laboratory, but effectively enhancing gold solid support known as Klarite, or colloid suspensions are now also commercially available.

Another approach to enhance Raman scattering is offered by tip-enhanced Raman spectroscopy (TERS), reported for the first time in 2000 [62] and significantly developed in the last ten years [63–67]. In TERS, very strong electromagnetic field is located at the nanometer-scale metallic tip (Ag or Au). In an ideal case, it ends with a single metal nanoparticle. Its radius is much smaller than the diffraction limit so it allows measuring the spectrum from a much more confined spot. The Raman signal is strongly enhanced when tip is brought close to the sample irradiated by the laser beam (the movement is controlled by atomic force or scanning tunneling mi-

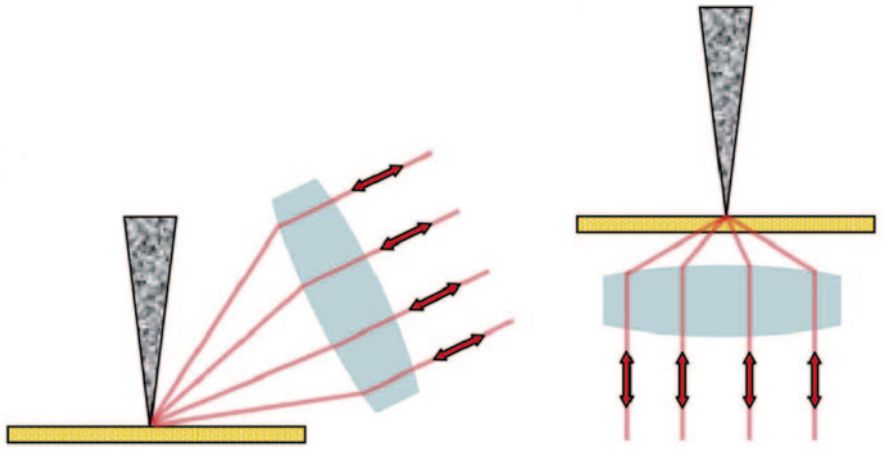

Fig. 3.4 Different configurations used in TERS experiments; *left*—side illumination, *right—bottom* illumination, using an inverted microscope. (Reproduced from Ref. [67] with kind permission of Elsevier BV)

croscopy head) (Fig. 3.4). Therefore, TERS provides spectroscopic characterization of the sample with a nanometric spatial resolution.

To date, many TERS experiments have been reported for a variety of chemical and biochemical systems. They have been thoroughly reviewed in 2008 by Bailo and Deckert [68]. In the last ten years, TERS spectroscopy has been significantly developed and opened new possibilities of applications in a variety of fields in biology and medicine. In particular, experiments with living cells seem to be very promising. Interesting review that shows the potential and challenges of TERS applications in bioscience was published in 2010 by Deckert-Gaudig and Deckert [69].

3.4 SERS in Biology, Biochemistry and Biomedicine

3.4.1 SERS of Aminoacids, Peptides, Proteins, and Enzymes

Applications of SERS in bioscience cover very broad area, from relatively simple systems such as aminoacids and peptides through proteins, nucleic acids and enzymes, up to individual cells, living tissues, bacteria and viruses. Advantages of SERS spectroscopy in structural studies of small, biologically important molecules such as amino acids, purine and pyrimidine bases, porphyrins, flavines etc. have been shown just after discovery of the SERS phenomenon. These early studies have been reviewed by Cotton in 1988 [70]. SERS of amino acids and proteins has been observed on various SERS active substrates, among which silver and gold colloids are the most important and the most popular. However, it is well known that

SERS spectra on metal colloids suffer from some irreproducibility [71–73] caused by aggregation of metallic nanoparticles. On the other hand, aggregating agents are indispensable to achieve better surface enhancement, because huge electromagnetic fields responsible for surface enhancement are created at particle junctions (nanogaps). A common problem in these experiments is the inhomogeneity of the colloids, which in turn provides local differences in the SERS enhancement factors. Most SERS studies of amino acids aimed at establishing the mode of interaction with the metal and the geometry of the molecule at the surface. SERS spectra allow determining the molecular form of amino acid present at the metal support (cationic, zwitterionic, or anionic), owing to high sensitivity of the spectrum to the molecular structure of the adsorbed species [74–79]. Some experimental SERS results are supported by theoretical calculations using DFT approach, making interpretation of the spectra more credible [80].

The most stable and reproducible SERS spectra of aminoacids have been recorded for sulphur containing molecules such as cysteine or methionine [77, 81–83]. SERS spectra of cysteine were recorded on various substrates such as colloidal nanoparticles [84] and rough silver electrodes, on which cysteine spontaneously self-assembles [81, 83]. Cysteine molecules are chiral—they can exist in one of two enantiomeric forms (L- or D-), that only differ in that their structures are mirror images of each other. Because only one enantiomer tends to be physiologically active while the other one is inactive or even toxic, drug compounds are produced in an enantiomerically pure form. Chiral surfaces may be applied as sensors for chiral recognition. In principle, both: the normal Raman and the SERS spectra are not sensitive to molecule chirality. However, in carefully designed experiments SERS spectra of cysteine monolayers on the Ag electrode surfaces have been shown to be sensitive to chirality of adjacent cysteine molecules at the metal surface [83]. It was demonstrated that there is a range of electrode potentials in which cysteine molecules are adsorbed mainly in zwitterionic form with the COO– groups close to the surface, while at more negative potentials NH_3^+ groups deprotonate at the surface with simultaneous weakening of the interaction of the carboxylic groups with the surface. As expected, the potential-induced effects for D-cysteine were similar to these for L-cysteine. However, for racemic mixture at acidic pH, the changes in the spectral pattern corresponding to potential-induced transition from adsorbed zwitterions to neutral molecule were considerably smaller. The origin of the observed effects were explained in terms of the most stable adsorption configuration for cysteine that involves metal-sulphur, metal-nitrogen and two hydrogen bonds between carboxylic groups (cyclic dimer) as indicated by DFT calculations [85]. In the case of LD cysteine dimers, there is a mismatch in the carboxylic bonds of the neighbouring molecules, which results in considerable weakening of the hydrogen bonds between them. The L- and D- cysteine enantiomers adsorb at the metal surface with equal probability, when adlayer is self-assembled from the solution. Thus, in spite of higher stability of the homochiral dimers, molecules of opposite chirality may exist at the surface in the immediate vicinity. In such a case intermolecular interactions between adjacent molecules are considerably weaker than for monolayer formed by the molecules of the same chirality, what is reflected in the band positions in the respective

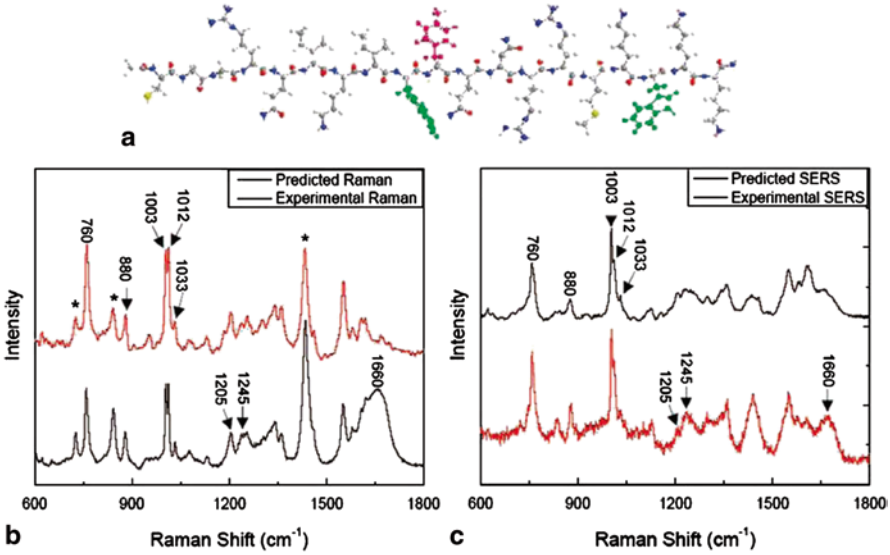

Fig. 3.5 Comparison of empirically predicted (*red*) and measured (*black*) spectra of penetratin. (**a**) Molecular model of penetratin, including one phenylalanine (*purple*) and two tryptophan (*green*). (**b**) Raman spectra (peaks assigned to tryptophan are denoted with an asterisk). (**c**) SERS spectra. (Reproduced from Ref. [91] with kind permission of the American Chemical Society. Copyright 2008, American Chemical Society)

SERS spectra. Another example that confirms sensitivity of the SERS spectra to chirality is recognition of phenylalanine enantiomers by the enantiomeric cysteine monolayer deposited on a silver support [86] and SERS evidence for chiral discrimination in intermolecular interactions between adsorbed methionine molecules [87]. Further exploration of the stereospecificity of SERS may open new possibilities of applications of SERS spectroscopy in optical sensors of chiral biomolecules.

TERS is a very helpful technique in investigating molecules of biological importance. In 2009, first TERS spectra of cystine adsorbed on ultrasmooth gold substrate were reported. They indicated local variations of cystine adsorption [88]. It was also possible to observe two differently protonated histidine forms and determine two different geometries of adsorption on an atomically flat silver substrate [89]. SERS data for simple amino acids made it possible to interpret the SERS spectra of more complex systems such as peptides [90–95], dipeptides [96, 97], and proteins [98–100]. For example, it was possible to predict major features of the SERS spectra of penetratin, a 19 amino acid cell penetrating peptide, based on the SERS spectra of aromatic aminoacids such as tryptophan and phenylalanine and cysteine-containing aromatic peptides bound to Au nanoshells [91]. As may be seen in Fig. 3.5, the SERS spectrum of penetratin is dominated by features characteristic of the aromatic amino acids, greatly simplifying its interpretation.

While interpreting complex SERS spectra of peptides and proteins, it must be kept in mind that the most strongly enhanced bands in the spectrum correspond to the vibrational modes involving part of the molecule in the vicinity of the metal

support. It has been proved that electromagnetic enhancement decays with distance from the surface, but is still measurable at about 10 nm [101]. Therefore, in case of very large biomolecules, spectral information may be confined to some fragments of the molecule directly interacting with the metal nanoparticle or in a short distance from the metal surface. This specificity and selectivity of the SERS spectra cause that in the case of large bio-molecules some fragments of the molecules are not "seen" in the spectra. On the other hand, this would enable characterization of the interaction between metal surfaces and biological molecule and determination of its orientation at the surface.

Also TERS spectra of peptides have been successfully recorded. TERS studies of peptide adsorption on metal surface (gold nanoplate) were carried out on a model molecule of oxidized glutathione and the structure of the peptide on the Au surface was determined [102]. The uniformity of the spectra measured at several points along a line across the gold substrate indicated the consistent orientation of the peptide. This result was regarded as crucial for possibility of characterizing and sequencing peptides with the help of this method.

Another problem related to SERS biosensing of proteins is their denaturation upon contact with metal nanoparticles. Thus the biocompatibility of the metal surface must be carefully controlled. The most simple way is to cover the nanoparticle surface with a self-assembled monolayer (SAM) [103] that prevents direct contact between the protein and the metal surface. There is a great variety of SAMs that could be used to prevent denaturation of the protein at the metal surface. However, the most optimal ones have to effectively bind the protein e.g. through electrostatic or covalent interaction, with simultaneous preservation of the protein properties and biological activity. The most popular SAMs are built of ω-substituted alkanethiols $HS(CH_2)_nX$, which form very stable metal-sulfur chemical bond. They create an interface with a well-defined composition and structure and with easily controlled properties achieved through the selective use of X groups. Among alkanethiols with negatively charged terminal groups, carboxylate-functionalized ones are the most frequently employed [104–109]. Sulfonate group is an example of an anionic group that can be used alternatively [110]. A great deal of work has been devoted to design biocompatible surfaces that ensure preserving the native structure of immobilized proteins. In the case of redox protein, communication between protein and electrode, which enables effective electron transfer, is also a major challenge. The case of cytochrome c (Cc) is a good illustration of the advantages for using SERS in studies of redox proteins. Numerous reports have been published on SERS of Cc immobilized on a silver or gold support modified with various monolayers [105, 108–112]. To improve intensity of the spectra, they are recorded using excitation laser line in resonance with molecular transition in Cc chromophore (SERRS spectra). The SERRS spectrum of Cc not only allows identifying the protein immobilized on the surface, but also makes it easy to determine the oxidation state and spin configuration of iron in heme groups, because Raman bands are well known markers of these properties [113, 114]. Moreover, sensitivity of the SERS spectrum to the orientation of the adsorbed molecules with respect to the surface causes that relative orientation of the heme plane may be determined by using so called surface

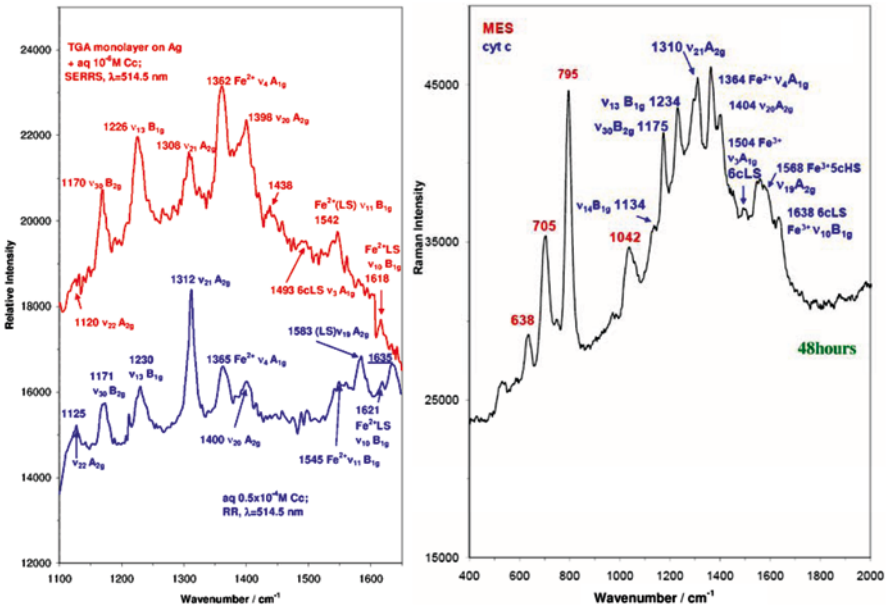

Fig. 3.6 SERRS spectra of cytochrome c adsorbed from 10^{-4} M aqueous solution of Cc on Ag electrode, coated with monolayer of thioglicolic acid (TGA) (*upper* spectrum—*left* panel) (A. Królikowska, unpublished results) and mercaptoethanesulphonate (MES) monolayer (*right* panel), as compared with the resonance Raman spectrum of 0.5×10^{-4} M Cc solution. (Reproduced from Ref. [110] with kind permission of John Wiley and Sons)

section rules [8, 115]. Figure 3.6 shows SERRS spectrum of Cc electrostatically immobilized on silver electrode, which has been covered with a monolayer of thioglicolic acid (TGA) and resonance Raman spectrum of Cc solution. As may be seen, both spectra exhibit different frequencies and intensity pattern of the bands, which excludes risk that the bands detected in the SERRS spectrum originate from the protein in the solution. The band positions in the SERRS spectra are characteristic of the native 6cLS configuration of heme iron. Namely, the band at 1362 cm^{-1} indicates Fe^{2+} oxidation state, together with the 1493, 1542 and 1618 cm^{-1} bands which are markers of the native low spin (LS) configuration. On the contrary, in some cases bands assigned to oxidized form of Cc (at 1568 and 1638 cm^{-1}) are clearly visible as for Ag support modified with mercaptoethanesulphonate (MES) (see spectrum in Fig. 3.6). The band at 1568 cm^{-1} indicates lots of Met-80 axial ligand leading to a non-native B2 5cHS state of heme and 1638 cm^{-1} feature is ascribed to the native B1 6cLS state [110].

Potential-dependent SERRS studies for Cc immobilized on functionalized SAMs on Ag allow monitoring conformational changes in interfacial redox processes [111]. SERRS experiments may be performed in the stationary conditions with continuously changing electrode potential [111, 116], as well as in time-resolved mode by applying a potential jump [117]. These spectroelectrochemical ex-

periments indicated that for the short thiol linkers the electron-transfer kinetics is determined by protein dynamics rather than by electron tunneling probabilities and that the reorientation of protein is controlled by the interfacial electric field. Thus, they show that SERRS can provide a very deep insight into dynamics of interfacial processes of proteins on the molecular level. SERRS experiments deliver much information about the heme part of the protein. A unique possibility of observing vibrational spectrum of both the heme and amino acid residues is however offered by TERS spectroscopy as demonstrated in 2008 [118].

Surface enhanced Raman scattering spectroscopy is now widely reported for enzymes. In this case, combination of SERS and resonance Raman spectroscopy is also usually applied, because it offers considerably higher sensitivity as compared to SERS. Excellent review on the development of SERS for the detection of enzyme transformations was published by Larmour et al. in 2010 [119]. Initially, SERRS was used for direct detection of enzymes. First results on SERRS of glucose oxidase has been reported at the very beginning of the SERS studies [120]. The first studies, in which biological activity of enzyme immobilized on Ag nanoparticles (Ag colloid) was monitored by SERRS spectroscopy, were published in 1993 [121]. In this report, 60–85 % retention of enzymatic activity of chlorocatechol dioxygenase in the reaction of catechol substrate with oxygen was demonstrated. The subsequent experiments for enzyme-substrate, enzyme-inhibitor, and enzyme-product complexes indicated that in general, SERRS allows probing the enzymatic processes *in situ*, during catalytic turnover. However, it has to be stressed that in all SERRS studies reported, some loss of the enzyme activity was observed upon contact with the metal support responsible for signal enhancement. Therefore, preparation of biocompatible substrates seems to be a key problem and important challenge in these experiments.

An alternative approach to study enzyme bioactivity with SERRS is recording the spectrum of enzymatically produced dyes, which are easily detected with SERRS. For example, azo dyes may be used in such experiments because they exhibit strong and very characteristic SERRS spectrum. SERRS signal of azoaniline adsorbed on Au nanoparticles has been applied for determination of glucose concentration [122], or adsorbed on Ag colloids for detection of antigen (mouse IgG) [123]. In this case azo dye was produced in peroxidase catalyzed oxidation reaction of o-phenylenediamine by hydrogen peroxide (Fig. 3.7).

The idea of utilizing SERRS spectrum of enzymatically produced dyes to detect enzymes was further developed by Stevenson et al. [124]. In a very interesting experiment, the SERRS spectrum of oxidized form of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), used as a substrate in an enzyme-linked immunosorbent assay (ELISA), was applied for the indirect detection of human prostate specific antigen (PSA) [124]. ABTS yields a green product upon enzymatic reaction with horseradish peroxidase and is commonly used as a colorimetric indicator of peroxidase activity. Experiments by Stevenson et al. [124] proved that SERRS provides a lower limit of detection and a wider range of linearity of the Raman signal vs. concentration as compared to the standard colorimetric ELISA approach.

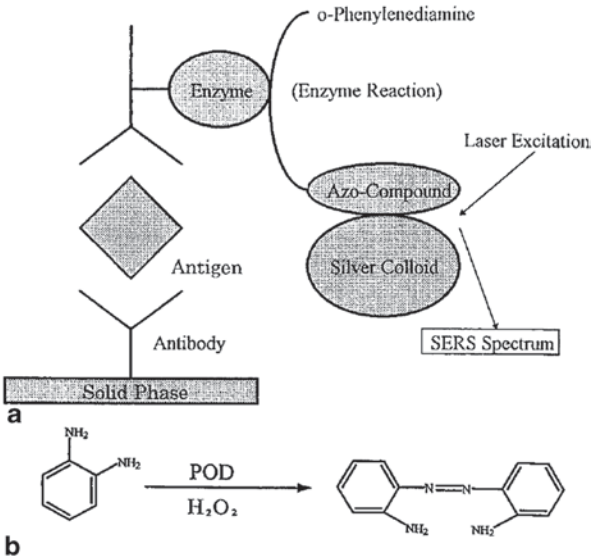

Fig. 3.7 SERS enzyme immunoassay system (**a**) and enzyme catalyzed reaction (**b**). (Reproduced from Ref. [123] with kind permission of the American Chemical Society)

Another example of successful application of SERRS for detecting the presence and measuring activity of the enzyme is the work of Ruan et al. [125], in which SERRS spectrum of a blue indigo dye produced by oxidation and dimerization of 5-bromo-4-chloro-3-indolyl phosphate was utilized for monitoring activity of alkaline phosphatase.

Detection of DNA is one of the most important tasks in bioanalysis. To date, numerous SERS measurements of DNA utilizing different SERS supports have been reported. Experiments can be roughly divided into two groups. In the first group, called label-free method, DNA is directly detected with the SERS nanoprobes. For example, Halas group reported SERS spectra of DNA oligonucleotides and found that they are selectively dominated by the vibrational modes of adenine [126]. The authors also demonstrated the capability of SERS in investigating the interaction of DNA with various molecules (in this case—cisplatin). While direct SERS measurements for DNA are rather scarce, there are many reports on the indirect SERS experiments. This approach to DNA detection consists of monitoring the SERS spectrum of reporter molecules (usually dyes) that are attached to the metallic nanoparticles. This kind of SERS experiments for DNA is more extensively described in sect. 4.2.1.

3.4.2 SERS Biosensors

Term "biosensor" has no clear definition. Therefore, it might be applied to many different systems. Generally, biosensors provide data about the biological analyte they are exposed to, monitoring a change in their physicochemical response. If we monitor a SERS spectrum coming from the sensor in varying conditions, we deal with a SERS sensor. There is a whole range of tasks which biosensors may be employed to: monitoring a chosen kind of molecules, identification of the analyte composition, or determination of the environment parameters. Previous section of this review describes SERS experiments used for detection of certain biomolecules such as proteins or enzymes. Those examples perfectly fit in what we call a biosensor—they give information about the presence of a chosen biochemical compound in the sample. Those SERS sensors make use of various types of metallic substrates to enhance Raman scattering. However, lots of opportunities in the cell analysis open if metallic nanoparticles are engaged: thanks to their size, they can be successfully introduced into the cell in order to report the intracellular conditions from a precisely defined place.

3.4.2.1 Specific Sensing of Biomolecules

Previously described experiments intended to detect biomolecules were carried out in the following manner: object of the study needs to be found in the close proximity of the Raman enhancing substrate and then its characteristic SERS spectrum is collected. There are cases, however, when such a direct approach cannot be applied. Problem of glucose sensing illustrates such situation perfectly. Glucose is a molecule of great importance in human body, especially due to its relation to diabetes which has been considered a disease of civilization in the Western World for years while its incidence in developing countries has been increasing rapidly. Monitoring a concentration of glucose with SERS is hindered by its small Raman cross section and minimal adsorption on SERS-active metals. Van Duyne et al. performed a series of experiments to improve and optimize sensing parameters of glucose [127–130]. They increased its affinity to the SERS substrate by adsorbing a SAM of decanethiol on the AgFON surface [127]. It let them detect glucose over a clinically interesting concentration range. Further investigations focused both on the composition of the partition layer and the substrate. Researches showed that the sensor with (1-mercaptoundeca-11-yl)tri(ethylene glycol) as a partition layer works also in presence of interfering specimens, such as bovine serum albumin [128]. Real-time sensing was possible with a mixed SAM consisting of decanethiol and mercaptohexanol [129]. Such a device exhibited stability for 10 days and was used to determine concentration of glucose in the environment of bovine plasma. On the other hand, it was proved that replacing AgFON with AuFON and 1-mercaptoundeca-11-yl)tri(ethylene glycol) with 1-mercaptoocta-8-yltri(ethylene glycol) resulted in red-shift of plasmonic resonance which could be a step forward towards apply-