Chapter 5
SERS for Bacteria, Viruses, and Protein Biosensing

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Abstract In this chapter, various techniques are reviewed with focus on the identification of complex biological agents such as bacteria, viruses, proteins, and enzymes using SERS-active silver substrates. Biological targets have multiple peculiarities that add to the challenges of the SERS biosensing. In regards to the direct non-labeled sensing of bacteria, it was discovered that all bands in the registered SER spectra were generated by metabolites released from bacterial cells. It undermined the prior notion of non-labeled detection and identification of bacteria based on the presumed spectra of cellular walls. However, it also provides new opportunities for the SERS analysis of bacteria. The SERS measurements of viruses can be performed with SERS-active surfaces or colloidal solutions of silver nanoparticles. However, the use of surfaces requires extensive sample preparation and often lacks sensitivity, while colloidal SERS substrates have another problem—most types of silver nanoparticles are negatively charged and have a poor interaction with likewise predominantly negatively charged virions. Thus, a challenge is posed to develop SERS-ready positively charged silver nanoparticles or use other methods to enforce the non-specific binding of viruses to the silver surfaces. Meanwhile, SER spectra of proteins are nearly impossible to acquire at adequate sensitivity. Thus, non-direct measurements are the only way. SERS provides the most benefits when working with relatively small molecules, so small molecules serving as Raman probes can be...
used as an intermediary to produce SER spectra. For enzymes like butyrylcholine-
terase, it means measuring SER spectra of substrates and products of the relevant reaction, while for other proteins, specialized techniques must be developed. It can be concluded that biological targets require a case-by-case approach. Prior experiences with direct SERS measurements of highly Raman-active molecules like R6G and others often used in fundamental studies might not be relevant in bioanalytics.

**Keywords**  SERS · bacteria · viruses · proteins · enzymes · biosensing

**Nomenclature**

4-mPBA  4-mercaptophenylboronic acid  
AChE  acetylcholinesterase  
AFM  atomic force microscopy  
AMP  adenosine monophosphate  
BChE  butyrylcholinesterase  
CDV  canine distemper virus  
EB-PVD  electron beam physical vapor deposition technique  
EF  enhancement factor  
ELISA  enzyme-linked immunosorbent assay  
EW  excitation wavelength  
GA  glycated albumin  
HSA  human serum albumin  
LDA  linear discriminant analysis  
LOD  limit of detection  
MYXV  myxomatosis virus  
PCA  principal component analysis  
PCR  polymerase chain reaction  
PLS  partial least squares regression  
PVX  potato virus X  
SEM  scanning electron microscopy  
SERS  surface enhanced Raman spectroscopy/scattering  
SER  surface enhanced Raman  
TCh  thiocholine  
TMV  tobacco mosaic virus  
TNB  5-thio-2-nitrobenzoate ion  
UV  ultraviolet  
VERS  volume-enhanced Raman spectroscopy/scattering
5.1 Introduction

Surface-enhanced Raman scattering (SERS) is a powerful analyzing tool providing the high sensitive detection of different types of molecular compounds (Sharma et al. 2012; Sarychev et al. 2019). SERS is promising in clinical diagnostics, which include the cancer detection and imaging, cancer therapy and drug delivery (Pazos et al. 2016; Oseledchyk et al. 2016; Litti et al. 2016; Schurmann and Bald 2016; Andreou et al. 2016; Chen et al. 2016b; Chen et al. 2016c; Li et al. 2016a, 2016b; Cheng et al. 2017; Kneipp 2017; Harmsen et al. 2017; Rastinehad 2019), quantitative control of glycated proteins for diabetes detection (Xu et al. 1999; Kiran et al. 2010; Dingari et al. 2012; Barman et al. 2012; Lin et al. 2014), cardiovascular biomarkers for early diagnosis of acute myocardial infarction (Das et al. 2008; Benford et al. 2009; Chon et al. 2014), etc.; in environmental and food safety for real-time monitoring of pathogenic bacteria, pesticides, and toxic molecules (Bodelon et al. 2016; Duan et al. 2016a; Duan et al. 2016b; Yang et al. 2016; Chen et al. 2016a; Zhou et al. 2016; Tian et al. 2016; Liu et al. 2017); for detection of ultra-low quantities of nerve gases, explosive substances, and other hazardous substances (Hakonen et al. 2016; Chen et al. 2017). The basis of the SERS sensing is the generation of several plasmon modes at a metallic surface and further Raman scattering of the plasmons by analyte molecules. The molecules are excited by surface plasmons and generate secondary plasmons which can be significantly enhanced. The radiation coming from these secondary plasmons produces a SERS signal (Brouers et al. 1997). Therefore, in SERS, the dominant contribution to enhancement factor (EF) is the electromagnetic mechanism (Ding et al. 2016) allowing it to reach very high sensitivity. This EF for SERS is evaluated as the fourth power of the intensity of the local electric field (Brouers et al. 1997; Itoh et al. 2017; Ding et al. 2017). Thus, the surface morphology of nanostructured SERS substrates is crucial for achieving high EFs. It is an important point in context of increasing the sensitivity of the biological and chemical sensing. Several groups have already demonstrated EFs from $10^4$ to $10^9$ for SERS substrates composed of clusters of gold or silver nanoparticles encapsulated in a dielectric matrix (McFarland et al. 2005; Perney et al. 2006; Yan et al. 2009; Fan et al. 2011; Banaee and Crozier 2011; Li et al. 2011; Mattucci et al. 2012; Huang et al. 2013; Hu et al. 2014; Lee et al. 2014; Zhang et al. 2015). Modern fabrication technologies such as focused ion-beam lithography, electron-beam lithography, X-ray, UV, and interference lithographies allow to produce SERS substrates with an accuracy control over the shape and spatial distribution of nanostructures. However, most of these techniques are still expensive and complicated. A large number of nanostructures such as nanodisks, nanoholes, and nanodimers have been tested and provided high EFs for SERS (Brolo et al. 2004; Lim et al. 2010; Suh and Odom 2013; Barbillon et al. 2019). High EF was also observed in a system of silver microplates resulting from the enhancement of the local electric field in gaps between plates (Nechaeva et al. 2020).

The chemical enhancement strongly depends on local electronic structures of the molecules and the substrate it interacts with as each of their wave functions begins
to overlap (Campion et al. 1995; Kambhampati et al. 1998; Otto 2005). Influence of chemical enhancement is significantly weaker than electromagnetic enhancement. Indeed, the magnitude order of the chemical enhancement is only $10^2$ (Jensen et al. 2008; Jahn et al. 2016), but it can play a crucial role in SERS. The maximum convergence of the analyzed molecules with the surface is extremely important.

In this chapter, we have reviewed useful techniques for identification of complex biological agents such as bacteria, viruses, proteins, and enzymes using SERS-active silver substrates.

5.2 Bacteria

Bioanalytical applications of label-free SERS for bacterial cells are fully defined by molecular origin of bacterial spectra. Early research papers on this topic (Picorel et al. 1990; Efrima and Bronk 1998; Zeiri et al. 2002, 2004) have shown that at excitation wavelengths (EWs) of 488 and 514.5 nm, SER spectra of various bacterial species, both Gram-positive and Gram-negative, are almost identical. Moreover, they do not differ from spectra of isolated cell membranes. Bands of oxidized or reduced forms of riboflavin as common components of cell membranes fully dominate in such spectra due to overlap of their extinction band with mentioned EWs, which leads to additional resonant enhancement of riboflavin spectrum. Such molecular origin of bacterial spectra at EWs of 488 and 514.5 nm holds the potential for their detection, but not discrimination of different species.

Pioneering studies on SERS of bacteria with longer EWs, particularly 785 nm, reported (Guzelian et al. 2002; Jarvis and Goodacre 2004; Premasiri et al. 2005) significant distinctive features in spectra of different species and sometimes strains. It has been hypothesized that label-free SERS of bacteria at EW of 785 nm might be used for both detection and identification of bacteria. Unfortunately, for more than 10 years, many attempts to assign observed bands to particular chemical substances were unconvincing as they were focused on single bands, but not the full spectrum (Guzelian et al. 2002; Jarvis and Goodacre 2004; Premasiri et al. 2005; Luo and Lin 2008; Kahraman et al. 2011; Feng et al. 2015; Su et al. 2015; Mosier-Boss 2017). However, it should be noted that adenine and guanine bands were identified in spectra (Guzelian et al. 2002; Luo and Lin 2008; Kahraman et al. 2011; Feng et al. 2015; Su et al. 2015), but their assignment to intracellular RNA/DNA raised questions about lacking bands of pyrimidines (thymine, uracil, and cytosine). Without solid basement of molecular origin, the main aim of the most bioanalytical papers was to demonstrate the ability of SERS to discriminate different species or strains using formal mathematical approach, based on application of principal component analysis (PCA) or similar procedures of dimensionality reduction to spectra, followed by discriminant or cluster analysis (Jarvis and Goodacre 2004; Patel et al. 2008; Sundaram et al. 2013; Mosier-Boss 2017; Witkowska et al. 2018).

Premasiri et al. (2016) decisively demonstrated that SER spectra of 10 various bacterial species at EW of 785 nm using “in situ” grown, aggregated-Au-nanoparticle-
covered SiO$_2$ substrate” originated from 6 purine derivatives (adenine, AMP, guanine, hypoxanthine, xanthine, and uric acid). It has been shown that these molecules were not bound to the cell surface, but were secreted by cells into the surrounding solution. Later the applicability of such assignment was confirmed for another type of SERS substrate, a sol of silver nanoparticles, and two EWs: 532 and 785 nm (Durovich et al. 2018). DH5α strain of Escherichia coli was used as a model bacterium; silver nanoparticles were incubated for 1 minute with thoroughly washed E. coli suspension followed by sol aggregation with NaCl for SER signal generation (Fig. 5.1). Bacterial SER spectra of a cell suspension batch showed high repeatability on a timescale of 10–20 min. However, during the course of 4 h of cell suspension storage, the overall intensity of spectra increased together with pronounced changes in intensity ratios of some strong bands (e.g., $I_{1325}/I_{655}$). Even more severe differences in spectra were observed for independently cultivated and harvested cells. Spectrum of the 0.22 mkm filtrate of cell suspension retained all the bands of the original suspension. Moreover, the intensities of these bands were substantially higher for filtrate. Thermal inactivation of cells at +90 °C for 1 h followed by washing resulted in almost complete disappearance of the spectra. They contained only 3 weak bands characteristic of intact bacteria (730, 1325, and 1450 cm$^{-1}$), the band of phenylalanine (1002 cm$^{-1}$), and two bands of the amide III (1230–1270 cm$^{-1}$) and amide I (1640–1680 cm$^{-1}$). Despite strong variations in spectra, they contained a limited number of bands. All of these bands both for the entire set of spectra or any particular spectrum were in a good agreement with positions and relative intensities of 4 purines: adenine, guanine, hypoxanthine, and xanthine. These substances were gradually released by cells into the surrounding media; their presence was characteristic of viable cells only; the ratio of purines varied both for different batches of the same strand and during 4 h storage in starvation conditions.

This newly revealed molecular origin of bacterial SER spectra at EWs of 532 and 785 nm considerably reshapes the entire bioanalytical area of label-free SERS of bacteria. Indeed, from this point of view, the problem of discrimination of bacterial species and strains is strongly related to dissimilarities in their purines metabolism. On the other hand, the presence of purines might be used as a general bacterial marker for their detection. It also starts a whole new field of SERS application for purine metabolome profiling in bacteria.
5.3 Identification of Animal and Plant Viruses

Viruses represent another type of biological object for SERS measurements. There is an urgent need to develop new rapid methods of virus detection, only further exacerbated by the emergence of new viral diseases, such as novel coronaviruses or new influenza strains (Afrough et al. 2019).

Highly sensitive and express techniques are thus in demand. Nowadays, there are two mutually exclusive types of solutions: highly sensitive techniques (variations of ELISA and PCR) and rapid techniques (such as immune–chromatography strips). The methods with sufficient sensitivity lack the necessary speed, which limits their applications in epidemics control. The rapid techniques mostly lack required sensitivity and cannot detect viruses without prior concentration.

SERS techniques appear to have almost the same limit of detection as ELISA in some cases, such as the SERS detection of porcine circovirus’ intact virions (Luo et al. 2013), influenza virus SERS assay (Kukushkin et al. 2019), hepatitis B antigen detection (Kamińska et al. 2015), and many others. Thus, SERS is considered to be one of the most promising approaches to achieve both rapid and sensitive detection of viral pathogens. Non-labeled detection, while harder to achieve, is especially alluring, due to the fact that it enables the detection of multiple different targets as well as new strains without the need for pre-existing antibodies or other selective agents. There are also less requirements for reagents and materials, and no time spent on any specific binding or amplification reaction.

There are several peculiarities associated with viruses in general, that must be taken into consideration before attempting to develop a SERS-based detection and/or identification methods. First of all, viral particles are almost always negatively charged due to low isoelectric point value, which lies around 4 to 5 for most viruses (Michen and Graule 2010). Simultaneously, many viruses are very fragile and stable only at specific pH values, which makes it impossible to reliably alter the charge of virions by adjusting pH of the solution. Most frequently used methods of silver nanoparticles synthesis produce negatively charged particles. Such particles have poor compatibility with negatively charged analytes. Another issue is the shape and size of virions. Most viruses can not fit entirely into hot spots of normal SERS substrates. That means that only surface components of the virions generate SERS signals, and the strength of that signal might be greatly reduced. While some viruses with thread-like shapes might generate strong SERS signals from both exterior and interior components, large spherical viruses might not. Finally, viruses might have empty virions (no nucleic acid inside) or destroyed virions’ fragments present in the solution, which further complicates data interpretation.

Taking into consideration the above-mentioned challenges, SERS-active surfaces remain the main focus for label-free SERS-based virus detection. One of the common approaches to solve the problem with the size of various viral particles is the application of nanocavities, which can incorporate the entire virion inside, maximizing the contacts between the virus and the hot spots (Chang et al. 2011; Yan et al. 2017). In fact, the entire internal volume of the nanocavities, depending on
their shape and size, might benefit from the electromagnetic signal enhancement, an effect which is sometimes called VERS (volume-enhanced Raman scattering) (Zhang et al. 2019), and can be used to acquire scattered light from the internal components of the large virions.

In one of our prior works (Durmanov et al. 2018), a SERS substrate with nanocavities was utilized for non-labeled SERS analysis of four viral species—two thin, thread-like non-enveloped plant viruses, and two large spherical enveloped animal viruses. The substrates were fabricated with electron beam physical vapor deposition technique (EB-PVD), achieving porous interwoven surface by altering temperature of the mica substrate and tuning adatom energy levels by altering EB acceleration voltage. EB-PVD, while not exactly precise, is a cheap and readily available method of fabrication. The resulting SERS substrates contained a large amount of nanocavities, 200–300 nm in diameter, as characterized by SEM and AFM methods. The 3-dimensional reconstruction, based on SEM and AFM images, can be seen in Fig. 5.2.

The tobacco mosaic virus (TMV) and the Potato virus X (PVX) are RNA containing plant viruses with a thin unenveloped thread-like capsid. Moreover, the two animal viruses, viz. Canine Distemper virus (CDV) and the rabbit Myxomatosis virus (MYXV), are large, with MYXV being somewhat larger, roughly spherical (CDV) or brick-shaped (MYXV), and enveloped, though their respective envelope-formation mechanisms are different. Also, one is a DNA-containing virus (MYXV), while the other RNA (CDV). The viruses were purified and diluted in the same buffer solution, and then deposited on the SERS-active surface and dried. Raman spectra were collected from the dried spot, containing adsorbed virions. The concentration was about 100 viral particles per the detection zone.

Then the preprocessing was applied: the spectra were trimmed to the region between 1800 cm\(^{-1}\) and 400 cm\(^{-1}\), and cosmic spikes were removed by straight line generation; then baseline was corrected, mean buffer spectrum was subtracted from each spectra, and vector normalization was applied. Figure 5.3 represents the averaged spectrum of each virus. For visual contrast, the values are also presented in a heat-map style.

![Fig. 5.2 Three-dimensional reconstruction of the SERS-active porous surface, based on SEM and AFM images](image)
The expected differences between viruses should be attributed to the variations in their structure and components. And indeed, the areas of interest, as indicated by the heat-map, correspond to the expected differences in chemical properties. The bands of cholesterol and other lipid content, as well as $\beta$-sheets typical for membrane proteins ($610, 1067–69, 1098, 1302, 1443, 1448, 1491, 1676, and 1734 \text{ cm}^{-1}$), are present in the spectra of MYXV and CDV, but absent in TMV and PVX, due to their lack of an envelope. Some bands have high influence in all groups: $770–800 \text{ cm}^{-1}, 1006–1008 \text{ cm}^{-1}, 1430–1450 \text{ cm}^{-1}, and 1650–1700 \text{ cm}^{-1}$. These might be related to proteins, nucleic acids, and aromatic amino acids. Some bands, like $1103, 1108, 1463 \text{ cm}^{-1}$, and others, are more pronounced in the two plant viruses. Presumably, these bands correspond to the nucleic acid components among other things. Since plant viruses have thin thread-like structure, their internal components, namely, RNA, are closer to the silver surface and thus can contribute more to the SERS signal. There are also differences between amino acid composition, seen at $\sim 480 \text{ cm}^{-1}, \sim 850 \text{ cm}^{-1}, and 1560 \text{ cm}^{-1}$.

The collation of PCA-LDA values, visually observed differences, and expected variation in SERS bands positions points to the conclusion that label-free SERS can indeed support the identification of viruses based on their spectra. The overhaul accuracy of the classification model, trained with the spectra of the viruses, was no less than 99.4%. The main challenge is sensitivity. One of the possible ways to increase it is to use SERS colloids, such as silver nanoparticles. The problem with the charge of the viruses might be overcome by using positively charged aggrega-

![Averaged spectra of each virus (black line). From top to the bottom: CDV, MYXV, TMV, and PVX. Each Raman shift is marked by a colored vertical line. The color of the line reflects the value of the averaged spectrum at the corresponding Raman shift. The values are colored by a gradient from dark red (the highest value within all averaged spectra) to the dark blue (the lowest value).](image-url)
tion reagents, which would act as intermediaries between negatively charged nanoparticles and virions. One example of that approach is the use of spermin, which was successfully applied to the nucleic acid SERS measurement (Li et al. 2018). The results for viruses are unclear so far, which opens a whole new field of research.

5.4 Quantitative Enzyme Detection

Since solid SERS-substrate application is limited by diffusion, it is very important to attract the analyte to the surface. Special attraction techniques can include imprinting in complex-shaped pores (Li et al. 2020; Ren et al. 2020), antibodies (Kamińska et al. 2017; Smolsky et al. 2017), or labels (e.g., peptides or low-molecular-weight targeting ligands (Lee et al. 2010; Li et al. 2016a, 2016b), or just utilize covalent bonding to the surface of the substrate (Shan et al. 2018). Here we describe two examples of covalent interaction with the view of analyte attraction.

Butyrylcholinesterase (BChE), or pseudocholinesterase, is a serine hydrolase closely related to acetylcholinesterase (AChE). This enzyme is of high toxicological and pharmacological importance because it hydrolyzes ester-containing drugs and scavenges cholinesterase inhibitors including potent organophosphorus nerve agents (Masson and Lockridge 2010; Lockridge 2015; Lockridge et al. 2016). Decreased BChE level is a diagnostic and prognostic indicator for organophosphate poisoning. BChE activity is associated with obesity (Li et al. 2008; Lima et al. 2013), with insulin resistance and the metabolic syndrome (Valle et al. 2006; Iwasaki et al. 2007), hyperlipidemia (Kalman et al. 2004), coronary artery disease and hypertension (Alcantara et al. 2002), and the arterial pathology of diabetes mellitus (Vaisi-Raygani et al. 2010). Thus, plasma level of BChE represents an important prognostic marker in the organophosphate exposure biomonitoring and in the diagnostic network of various patient clinical conditions.

One of the most widespread techniques for estimation of the cholinesterase activity is the detection of thiocholine—the product of enzymatic hydrolysis of butyrylthiocholine. Comparing to electrochemical methods (Arduini et al. 2009; Eremenko et al. 2012; Sgobbi et al. 2013; Kurochkin et al. 2014), Raman spectroscopy detection has a number of advantages: since the sample can be dried, the technique is suitable for routine measurements, the experiment can be carried out far away from the special equipment, and it is not necessary to transport blood or plasma samples.

Preparation and characterization of silver SERS-substrate were described by Nechaeva et al. (2018). In this study, silver-containing polymeric paste was applied to a 5-mm thick aluminum block and dried. The surface represents heterogeneous flaked structure with silver particles of various sizes and shapes. Numerous points of contact as well as the variation of the angles of incident light make possible the local enhancement of electromagnetic field, which causes a gain of Raman signal of the analyte.
The silver SERS substrate was used for thiocholine determination. Thiocholine solution was obtained by the enzymatic hydrolysis of butyrylthiocholine chloride according to the Scheme (1). The concentration of produced TCh was determined by Ellman's assay (Ellman et al. 1961). The conversion from the substrate reached 100%. The thiocholine concentration was calculated by the Beer–Lambert–Bouguer law ($\varepsilon_{412} = 14.15 \text{ ml/\mu mol} \cdot \text{cm}$ is the absorption coefficient of 5-thio-2-nitrobenzoate ion (TNB) at 412 nm) (Eyer et al. 2003).

The obtained Raman spectra of thiocholine have several characteristic peaks, the most intensive 773 cm$^{-1}$ can be attributed to CH3 rocking vibration modes (Liron et al. 2011).

For different concentrations of TCh, the resulting spectra have different intensities of 773 cm$^{-1}$ peak (Fig. 5.4). Therefore, it is possible to plot a calibration curve based on this peak. Each point at this curve represents the average of 10 replicates of TChCl spectra, and zero-point means phosphate buffer. The right axis on this figure demonstrates the PLS-calibration curve of thiocholine, the reference was Ellman's method. It is possible to calculate the limit of detection (LOD) for TCh

![Fig. 5.4](image)

**Fig. 5.4** The calibration curve of thiocholine chloride on the silver SERS substrate based on intensity of 773 cm$^{-1}$ peak (black) and PLS-regression plot for the same data (red). The inset shows dependence of 773 cm$^{-1}$ peak intensity from the concentration of TCh, concentrations from the top to the bottom: 50, 30, 20, and 5 $\mu$M
using a calibration curve. For developed technique, LOD (TCh) is 260 nM and sensitivity is 103.92 intensity units/1 μM.

It was shown that BTCh presence does not affect TCh detection on silver SERS substrate (Nechaeva et al. 2018). This conclusion is very important because thiocholine is the product of enzymatic hydrolysis of butyrylthiocholine (Scheme 5.1). The resulting curve at the end of the enzymatic reaction enables us thiocholine determination (and BChE activity correspondingly) despite the BTCh presence.

Butyrylcholinesterase activity in solution is strongly connected with thiocholine concentration and can be evaluated by measuring TCh. The solutions with high initial BChE activity give intensive Raman spectra because of high concentration of the TCh, and the solutions with low BChE activity contrariwise give low-intensive spectra. As for TCh, BChE calibration curve in the buffer and in human plasma can be created in two ways—by 773 cm$^{-1}$ peak and by PLS regression. The offset for BChE calibration curve in plasma also takes into account initial BChE contained in human plasma. It must be highlighted that the matrix effect does not impact the results of measurements.

Silver SERS substrate can be applied for low-molecular substances determination, for example, thiocholine, for enzymatic activity detection in model systems and in spike solutions with plasma. This technique has a number of undeniable advantages: it is label-free, inexpensive, and has the possibility to routine measurements of hundreds of samples.

### 5.5 Glycated Human Albumin Biosensing

The SERS substrate described above can be also used for glycated albumin (GA) biosensing. GA level shows the average concentration blood sugars over 2 weeks (Koga et al. 2010). Thus, GA can be considered as a marker for glycemic status (Kohzuma and Koga 2010). The present techniques of GA determination often require antibodies and enzymes and also represent many-step difficult processes. We propose to expand the possible methods of analyzing the content of GA in plasma by using the SERS effect. There are a number of previous studies available which demonstrated the prospective use of SERS method for plasma glucose monitoring (Chen et al. 2011; Sun et al. 2014a, 2014b; Usta et al. 2016; Zhao et al. 2017).

The analysis itself includes silver SERS substrate, described above (Fig. 5.5a), modified with low-molecular-weight SERS label 4-mercaptophenylboronic acid.
This label creates very strong covalent bond Ag–S with silver surface and on the other hand has affinity to cis-diols and polyols due to boronic group. Surface modification of SERS substrate with 4-mPBA helps to produce specific substrate for cis-diol and polyols (including glycated proteins). Nechaeva et al. (2020) proposed the use of 4-mPBA-modified silver SERS substrate for one-step GA measuring both in the buffer and human plasma. In this work, selective changes of SERS spectra have been observed due to ionization of 4-mPBA on the surface. The main differences are observed at 416, 470, 999, 1021, 1072, 1572, and 1589 cm$^{-1}$ due to symmetry breaking of a 4-mPBA molecule from nearly C$_2$v to C$_s$ (Sun et al. 2014a, 2014b; Su et al. 2017). An obvious change of 8a (1586 cm$^{-1}$) and 8b (1573 cm$^{-1}$) modes is observed (Fig. 5.5b). Peaks demonstrate different areas, maximum position, different total intensity, and intensity of shoulders. All these changes indicate the difference between molecular HS-Ph-B(OH)$_2$ and anionic HS-Ph-B(OH)$_3$ forms of 4-mPBA.

4-mPBA band amplitude is distributed unevenly on the surface due to the heterogeneous morphology. Maximal Raman signals correspond to the excitation of gap plasmons between silver flakes as it is discussed above.

Silver SERS substrate modified with 4-mPBA was applied for sugars and glycated albumin determination. This technique provides detection of only covalently bonded molecules. To explore minor differences appearing due to glycation, statistical methods were used. Principal component analysis (PCA) was used to distinguish sugar spectra. The main differences that help to distinguish sugar spectra from the control lay in ranges 400–500 cm$^{-1}$, 950–1100 cm$^{-1}$, and 1550–1630 cm$^{-1}$ and correspond to 4-mPBA vibrations including in-plane benzene ring breathing mode and totally and non-totally symmetric ring-stretching vibrational modes.

For distinguishing human serum albumin (HSA) and glycated albumin (GA)—proteins with very similar structure—PLS (partial least square) regression was used. First, a model for standard concentrations of mixture HSA + GA was built. GA fraction was 24, 48, 60, 90, and 120 μM, and HSA was used as control (0 μM of GA). Then, using this model for GA, we can predict the value of GA in unknown samples. Plasma from three different healthy donors was examined on the SERS

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**Fig. 5.5** (a) SEM image of silver SERS-substrate surface, (b) comparison of Raman spectra of anionic and molecular form of 4-mPBA
substrate modified with 4-mPBA. The most significant loadings represent Raman shifts corresponded to 4-mPBA vibrations (ranges 250–450 cm$^{-1}$, 1000–1100 cm$^{-1}$, and 1600–1650 cm$^{-1}$) as in case with sugars. Although human plasma contains different sugars, they don’t affect the protein detection. We trained our model on protein spectra, thus we take into account only bands that play a key role in protein distinguishing. Predicted GA values are consistent with independent laboratory values. Thus silver SERS substrate modified with 4-mPBA allows quantitative biosensing of GA in the buffer and human plasma.

This technique requires an extremely low volume of the sample—about 15 μl—to measure 4 replicates. The method simplifies the experimental procedure and can be automated by computer processing of measurements. It also has an important advantage of technical simplicity since our silver-based substrates are easy to manufacture and operate.

5.6 Conclusion

Biological targets often differ from expectations. The revelation about the true nature of the SER spectra of bacteria came after more than a decade of multiple publications in which researchers demonstrated their ability to acquire SER spectra of bacterial cells, and used it to form classification models that successfully identified bacteria down to a strain. However, their results naturally couldn’t be reproduced in another lab, since every time the metabolites would be present in slightly differing proportions, and the classification model would have to be formed anew. That long-lasting confusion stemmed from a rigid approach and treating a complex living target like a static chemical reagent. Researchers expected to see the repeat of the same logic they observed when working with classical Raman probes like R6G and others. Viruses proved to be likewise tricky. It remains unknown what viral components actually provide the majority of the SERS signal, and the difficulties associated with the sample preparation render that field of biosensing impractical so far. Colloidal solutions can be an answer to some of those concerns, but also pose a number of new challenges caused by the charge interaction and the presence of various impurities. As for the proteins, non-direct measurements seem to be the main trend since protein molecules usually do not produce strong-enough SERS signals. But non-direct methods cannot be universal and applicable to a large variety of different proteins and have to be developed case-by-case. Thus, in order to create new biosensing techniques, more fundamental studies are a necessity.

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