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Abstract

Testing for the presence of microorganisms in biological samples in order to diagnose infections is very common at all levels of health care. There is a growing need to ensure appropriate diagnosis by also minimizing the analysis time, both being very important concerns related to the risk of developing an antimicrobial resistance. Moreover, there are important medical and financial implications associated with infections. In this chapter, we will discuss the latest ultrasensitive and selective, but simple, rapid and inexpensive bacteria detection and identification methods by using receptor-free and innovative immobilization principles of the biomass. Raman spectroscopy, which combines the selectivity of the method with the sensitivity of the surface-enhanced Raman scattering (SERS) effect, is used in correlation with chemometric techniques in order to develop biosensors for pathogenic microorganisms.

Keywords: surface-enhanced Raman scattering (SERS), single-cell detection, label-free, principal component analysis (PCA), biosensors

1. Introduction

Lately, the pathogens can be individually identified by using surface-enhanced Raman scattering (SERS), without the need of labeling or specific receptor usage like antibodies, for instance. Colloidal metallic suspensions offer the advantage of ambient conditions, fast completion, and minimal number of reactants, being economical, and resulting in a ready-to-use product. However, despite the progress achieved, concerns and problems with the preparation of metal nanoparticles (NPs) remain, such as the byproducts from the reducing agent, the multiple steps...
often required, and the high concentration of protective agents. Furthermore, it has been a major bottleneck to elucidate the key factors (other than surface roughness enhanced electromagnetic fields) that play important roles in the SERS process of adsorbed biomolecules. The understanding of the mechanisms involved in the interaction of biological systems with inorganic materials is of interest in both fundamental and applied disciplines. Herein, the decisive know-how in investigating biological samples by using several SERS-active platforms will be described.

1.1. SERS effect

Raman spectroscopy requires the illumination of a sample with monochromatic light. The inelastic scattering of a small fraction (approximately one in a million) of the incident photons toward lower (Stokes scattering) or higher frequencies (anti-Stokes scattering) than the incident light is known as Raman scattering. A typical Raman spectrum plots the intensity of the scattered light versus the number of probed molecules. There are several noteworthy advantages of this technique, such as speed, versatility, and the functionality under ambient conditions in nonspecific environments (by using portable, miniaturized spectrometers); the simplicity of sample preparation; the possibility of remote detection of Raman signals by using optical fiber probes; the chance to examine transparent samples; and the obliviousness to water, ubiquitous element for biological samples. Probably the biggest disadvantage lies in the extremely small cross section, typically \((10^{-30} - 10^{-25}) \text{cm}^2/\text{molecule}\), which can be translated into long acquisition times and considerable high sample concentrations.

Raman spectra can be used for the identification and classification of microorganisms once a procedure with good reproducibility and reliability is established [1–4]. However, the spontaneous Raman effect is so weak that fluorescence, when it occurs, obscures the Raman spectrum. SERS represents the enhancement of Raman-active vibrations associated with the intimate contact (within few nanometers) to a surface covered with plasmonic NPs. Moreover, additional modes not found in the traditional Raman spectrum can be present in the SERS spectrum, while other modes can disappear.

The surface-selection rules that apply to infrared and Raman spectroscopies are extended for surface-enhanced vibrational spectroscopy (SEVS) by taking into account the local field and/or the roughness of the surface. SEVS spectra are the expression of the analyte-radiation interaction when the molecule is in the close proximity or adsorbed on the metallic nanostructure, which supports the surface plasmons [5]. So the presence of the plasmon resonance, for instance, will define the observed spectral intensities. When electromagnetic radiation with the same frequency is incident upon the nanostructure, the electric field of the radiation drives the conduction electrons into collective oscillation. Electromagnetic enhancement, the major contribution in the SERS effect, relies on the Raman-active molecules being confined within large electromagnetic fields (EFs), generated by the excitation of the local surface plasmon resonance (LSPR). So the extreme sensitivity of SERS to small increases in the local field is easily seen since it scales roughly as \(\omega^4\) (where \(\omega = \text{frequency}\)). Therefore, the fall-off in intensity of high frequency vibrations is also explained; the driving field and scattered field cannot simultaneously excite the particle resonance if they are of very different frequencies. This explains also
the different excitation profiles for different bands; maxima for higher frequency vibrations occur at shorter wavelengths, as the scattered field is brought closer to resonance [6].

SERS represents a relatively inexpensive alternative, compared to the conventional detection methods that also meet the clinical tools’ requirements: simplicity, reliability, uniformity (for testing various pathogens), and high specificity. It completely overcomes the shortcoming of Raman small cross sections. SERS is capable to characterize [7–10], identify [11, 12], and differentiate [13, 14] pathogenic microorganisms in synergy with chemometrics, based on the biochemical, chemical, and their structural properties.

Even though SERS is a highly specific and sensitive detection method, well suited for biological issues, SERS measurements still suffer from low reproducibility of spectra. Fluctuations of spectral characteristics are induced by variation between different colloid batches, colloid concentration dependence, and inconsistent enhancement even within one colloid batch mainly due to an inhomogeneous and a rather uncontrollable aggregation of NPs [15]. The main issues consist in the difficulty to generate uniform distributed EFs, large EFs occurring only at localized positions (hot-spots) and the polydispersity of colloidal clusters. As Nie and coworkers [16, 17] have already quite convincingly demonstrated, the enhancement factor depends on the wavelength of exciting radiation, or rather on the relation between the wavelength and the size of the Ag NPs.

Still, for real-world applications, reproducibility is considered in particular cases more important than enhancement factors. Background signal from the food and environmental matrices represents a real challenge. In addition, proper and simplified sample pretreatment is needed before conducting a SERS measurement. For instance, sample preparation for SERS detection of bacteria is quite inconsistent referring to colloids as SERS-active substrates. The NPs can be either coated on the outside of the bacterial cell wall or directed to the interior of the bacterial cells. Whereas the first preparation results in spectral information mainly derived from cell wall components, the second one contains additional cytoplasmic information [18, 19]. Figure 1 shows the SERS signal acquisition process from a microbiologic sample, when the silver coverage of the bacteria (in blue) is successful.

Conclusively, the SERS effect depends on a wide range of parameters, such as the particular features of the laser excitation (wavelength, polarization, and angle of incidence), the experimental setup (scattering configuration), substrate-related parameters (geometry, adsorption, orientation with respect to the incident beam direction, and polarization), and is distance-dependent. However, readiness remains an important parameter in choosing the suitable, fast, and reliable tool for detection at trace level, for large-scale applications.

1.2. Gold or silver NPs in biomedical applications?

Gold NPs (Au NPs) are promising SERS candidates in biomedicine and have already been successfully tested for various biomedical applications. They are easy to prepare, significantly more stable than other metallic NPs (not easily oxidized), and are highly biocompatible. They can act as artificial antibodies due to their simple surface chemistry, precise binding affinity, and possibility of tuning by varying the density of ligands on their surfaces. Lately, a continuous effort was made to develop new low-cost and easier synthesis strategies for increasing
their cellular biocompatibility, by varying their geometries, their physical dimensions, and functionality. The mixing rate of the reactants could greatly influence the physical properties of the Au NPs, their stability over long periods of time, and their SERS sensitivity. It is reported that when the gold salt solution is rapidly added to the reaction mixture, preponderant spherical short- and long-chain polyethylene glycol (PEG) Au NPs with a mean diameter of 15 nm are obtained, whereas a drop-wise addition of the gold salt leads to a seeding effect and to Au NPs with a mean diameter of 60 nm [20]. The most common surface ligand used in biomedical applications is thiolated PEG (PEG-SH), which ensures the desired hydrophilicity and increased circulatory half-life in vivo systems [21]. Proteins, such as bovine serum albumin or collagen, can also serve as capping and stabilizing agents in the one-step synthesis of gold colloidal nanoassemblies and spherical Au NPs with tunable shape and size [22, 23]. Furthermore, the in vitro uptake and toxicity effect of Au NPs grown with a native collagen shell exhibit a lower toxic effect on cervical carcinoma and lung adenocarcinoma cells than synthetic polymer-coated Au NPs [24]. Additionally, due to their ability to efficiently convert light into heat, gold NPs can specifically allow thermal ablation of the targeted biological region and by absorbing high amounts of X-ray radiation become enhancers in cancer radiation therapy or computed tomography [21].

However, silver NPs (Ag NPs) show stronger plasmon fields than Au NPs due to the simple fact that their plasmon band does not overlap with the interband electronic transitions, as in the case of Au NPs [25]. Figure 2 presents our recent results obtained by using different SERS-
active substrates for the detection of E. coli. We synthesized several types of Ag and Au sols by using PEG with two chain lengths as reducing agent [20] and compared the obtained SERS signals with the in situ synthesized Ag NPs SERS signal of bacteria. The SERS spectra were recorded using a Raman microscope (Lab RAM HR, HORIBA Jobin Yvon, Japan). The 633-nm line of a HeNe laser was used as the excitation source. The excitation wavelength dependency of the SERS enhancement can be explained by the optical absorption of the silver colloidal suspensions. We already characterized the herein used NPs in our previous work; the Ag Hya NPs particles feature a plasmon resonance band of around 402 nm. Hence, excitation with the 532 nm laser line is favorable as compared to longer wavelengths (633 nm), being closer to the plasmonic band. However, in the case of the Au PEG NPs the plasmon resonance band is specific to gold and was determined around 540–560 nm, depending on the particles’ diameter. Generally, we waited 30 min as incubation time in order to obtain the higher and more stable SERS signal from the irradiated sample. As an effect of incubation time we observed an increasing agglomeration of the SERS-active colloids, leading to a coupling of the plasmon
resonances, which again leads to a red-shift of the main absorption. In consequence, we achieved in these earlier works the best SERS performance with the 633 nm excitation wavelength. As an indicator we selected the intensity of the marker band found at 732 cm$^{-1}$ which was about fivefold higher for the in situ approach. Even when we preconcentrated the Au PEG NPs by centrifugation (38× more concentrated Au colloidal suspension), the enhancement factor was not comparable with the one obtained for the hydroxylamine reduced in situ Ag colloid [12]. Moreover, the single-cell detection of bacteria was successfully obtained by using the in situ method, while the other tested colloids enabled us to detect bacteria only in rather high concentrations (>10$^3$ CFU/ml). For single-cell detection assays, this aspect could make a difference in selecting the SERS detection platform.

2. Label-free SERS-based assays

The impact on the public health demands sensitive analytical tools for detecting pathogens. Rapid, culture-free, ultrasensitive pathogens’ detection and identification are of paramount importance, since there are infections caused by a single microorganism (mycobacteria) and some pathogens need 20 days to proceed through one division cycle (while some *E. coli* strains take only 20 min), making laboratory culture a slow process.

Conventional methods currently used for microorganisms’ identification are nucleic acid-based polymerase chain reaction (PCR, qPCR, and real-time PCR), on-chip nucleic acid amplification [26], enzyme-linked immunosorbent assay (ELISA) [27], chemiluminescence-based microarrays [28, 29], and matrix-assisted laser desorption/ionization (MALDI, MALDI-TOF) [30, 31]. Major drawbacks of these culture-based detection techniques are the time required, the high costs, the need of prelabeling, and/or use of antibodies or DNA sequencing, and also the concerning increased rate of false negatives and false positives. In addition, biosensors for bacteria detection still rely on the specific capture of the targeted pathogen by using antibodies [9, 11, 32], aptamers [33], and substrates that contain metallic nanoscupltured thin films [34], or other different complex surface morphologies fabricated by using photolithography combined with deposition techniques [35]. This approach leads to costly microarrays, which can only be handled by trained personnel, in laboratory conditions. However, before any of these whole-organism fingerprint techniques can be used to analyze the samples, the microorganisms must be cultured in order to isolate the microorganism of interest from other sample constituents and/or produce sufficient biomass for analysis.

Recently, spectroscopic techniques look more and more promising with the development of low-cost, label-free, and ultrasensitive detection protocols enabling for the first time to be fast, specific, and sensitive enough in vital issues as healthcare. Particularly, Raman spectroscopy is a nonintrusive in situ analysis method, requiring small efforts for sample preparation and can be easily used outdoors with portable, miniaturized, and even handheld Raman spectrometers. Moreover, when using SERS, the spectral fingerprint reflects the physiological state of a bacterial cell, e.g., when pathogenic bacteria were cultured under conditions known to affect virulence, their SERS fingerprints changed significantly. It is also known that bacteria respond to environmental triggers, such as temperature, pH, and nutrient concentrations, by switching
to different physiological changes in their biochemical profile, including the number and composition of outer membrane proteins, lipopolysaccharides (LPS), and cellular fatty acids [36].

For SERS detection of bacteria, several innovative approaches are reported. Sengupta and coworkers [37–39] reported straightforward analysis of a colloidal-bacterial mixture in an optical glass cuvette. The preferred excitation laser line is 514.5 nm in their study of the pH influence and the time-dependent behavior of colloidal-bacterial suspensions, even if this wavelength is too long to resonate with excitations of the aromatic ring breathing mode.

By using the same excitation wavelength, Kahraman et al. [40] developed a uniform bacterial sample preparation method based on the convective assembly. Aggregation and clustering was frequently applied for obtaining higher SERS signal from “hot-spots” [41]. Knauer and others [9, 11, 42] optimized the microarray detection of single-bacterium by using different Ag sols and aggregation with sodium chloride or sodium azide in low concentrations. However, in these studies, the 633 nm laser line was selected for SERS-based detection on the antibody-activated microarray and the substrate used for enhancement was an Ag colloid produced by using a modified Leopold and Lendl method [43].

Efrima and Zeiri also proposed a novel approach, to use colloid produced in the presence of the biomass [18, 19]. The authors used the 633 nm laser line as an excitation wavelength, therefore they were able to report the ring breathing mode band observed at 1004 cm$^{-1}$ and assigned to the phenylalanine residue [10]. Excepting Knauer’s group work [9, 11, 42] and recent studies reported by Zhou et al. [12–14, 44], when applying the in situ approach, the Leopold and Lendl SERS active substrate was not so exploited in the bacteria detection, as the usage of the 633 nm laser line is mere. The hydroxylamine-reduced silver colloid was shown as ideal for obtaining SERS structural information on biological molecules contained in the bacterial cell wall, since it provides a high enhancement factor and shows almost no anomalies in the spectral band position upon aggregation [45] in comparison to the citrate-reduced Ag sols.

Another bacteria detection assay reported used crystal violet (CV) as Gram stain [46]: the procedure involved staining bacterial samples with CV which binds to the peptidoglycan layer of the Gram-positive and Gram-negative bacteria. Despite the simple and robust methodology of staining, the detection relies on optical microscopy, which is often susceptible to user-dependent sampling error. Therefore, by developing magneto-fluorescent NPs, the detection was improved and was successfully tested for both Gram-positive and Gram-negative bacteria (E. coli and S. aureus).

Label-free SERS-based detection is a very promising alternative for rapid monitoring real samples, offering single-cell sensitivity [1, 47], providing spectra with no contribution from the aqueous environment (prominent in the biological samples), and a high precision classification of bacteria, at strain level [1–3]. Recently, innovative approaches for the rapid SERS label-free detection of bacteria were developed:

(i). Simple, receptor-free immobilization of bacteria on the glass surface [14]. Miricescu et al., based on molecular-specific SERS spectra of uropathogens at single-cell level, discriminated
between rough and smooth strains of *E. coli* and *P. mirabilis*. The innovative and effective principle of bacteria immobilization through electrostatic forces, by inducing a positive charge on the silanized microarray surface was demonstrated for Gram-negative bacteria. In addition, the monitoring of single-cell SERS spectra of bacteria in different growth phases was assessed. (ii) *In situ* silver NPs preparation in the presence of bacteria (*Bacteria*® Ag NPs) [12]. Zhou et al. developed the *in situ* Ag colloid synthesis in two steps, resulting in coating the bacterial cell wall with a silver SERS-active layer. The assay requires about 10 min and only one sample droplet of 3 µl. By using this novel strategy, SERS detection (about 30-fold higher enhanced SERS signal) and hierarchy cluster analysis (HCA) discrimination of three strains of *E. coli* and one strain of *S. epidermidis* was reported.

2.1. *In situ* Ag NPs synthesis: extended approach

Currently and also in the future, biosensors with integrated nanotechnology promise to address the analytical needs in practical pathogen diagnosis. Recently, comprehensive reviews concerning the bacteria detection by using Raman and SERS spectroscopies were reported [15, 48, 49]. The increased sensitivity and high information content of SERS is acknowledged, mostly when this powerful tool is used in conjunction with advanced analysis and classification techniques. The key advantages that SERS-based biosensors include are the easy-to-use detection platforms and reduced testing time resulting in immediate diagnostic (within 5–15 min) [50, 51], superior sensitivity and multiplex capability [52], reduced sample volume, and high sensitivity and specificity. Thus, a great deal of research has been invested into the development of SERS-based biosensors for pathogenic microorganisms.

For instance, SERS mapping by using Ag dendrites [53] as SERS active substrate, both for Gram-negative and Gram-positive bacteria was reported. Not so promising results were obtained in case of the Gram-positive bacteria, probably due to their different membrane structure, containing less outside proteins. Usually, the marker bands used for detection of the pathogens are either the 1332 cm\(^{-1}\) band assigned to the CH deformations in proteins [53], either the 730 cm\(^{-1}\) band assigned to adenine [14, 54].

In this section we will mainly focus on the latest studies involving *in situ* Ag NPs synthesis approach for SERS detection in biomedical applications. In a more recent study, Zhou et al. [13] applied the *Bacteria*® Ag NPs approach for live and dead bacteria counting/discrimination.

![Figure 3](image_url)  
*Figure 3.* Scheme describing the *in situ* AgNPs synthesis on the bacterial cell wall.
Moreover, by using antibiotics (ampicillin, polymyxin B, and chloramphenicol) with different action mechanisms on bacteria and by monitoring the SERS signal decay in time, they were able to determine which microorganism is or not developing a drug resistance in less than 4 h. Lately, the same group [44] completed the previous work by applying the Bacteria® Ag NPs approach on a microarray (containing antibodies). Figure 3 exhibits the schematic protocol of generating the Bacteria® Ag NPs for SERS detection of microorganisms at single-cell level.

Since Efrima’s group reported on producing in situ NPs through external (bacterial cell-wall) or internal (interior components mode) synthesis on bacteria [18], the use of in situ synthesized Ag colloids for bacteria detection was demonstrated in several assays only by Haisch’s group [12–14, 44, 54].

Recently, a label-free NIR-SERS detection and discrimination of bacteria after pretreatment of bacterial cell membrane with disrupting agents was presented, featuring a sensitivity down to $10^3$ CFU/ml and a measuring time of less than 5 min [55]. Latest studies underline the applicability of the in situ synthesized Ag colloids also in environmental research, for instance, for the detection of bacteria in plant roots [56] and for pesticide monitoring in spinach leaves [57]. These results demonstrate the applicability of SERS-based noninvasive detection approaches for identification and characterization of pathogens and their secreted metabolites. The in situ synthesis of Ag colloid ensures the structural integrity of the coated bacterial cells and thus helps to rapidly generate spectral bacterial signatures with high sensitivity and specificity. Figure 4 contains a collection of microscopic images illustrating the reproducible coverage of E. coli cells with a silver layer by using the in situ synthesis approach.

Figure 4. Microscopic 100× images showing the Ag NPs coverage of bacteria (E. coli) when using the in situ synthesis approach (Bacteria® Ag NPs).
The influence at strain level of the O-antigen presence was already demonstrated by using unspecific surface chemistry as means of bacteria adsorption and the in situ synthesis approach [14]. O-antigen is the terminal structural part of the Gram-negative bacterial outer membrane that contains the significant variation between virulent strains and lab-designed strains. The differences in the composition of the monosaccharide units and sugar linkages translate into strain discrimination, by using molecular serotyping tests and chemometric analysis. Considering all this, O-antigen is the most variable cell constituent and of great importance when dealing with bacteria identification at strain level.

Raw SERS spectra collected from single cells of four different strains of *E. coli* are shown in Figure 5. For the accurate detection and discrimination between these strains, one has to make sure that all experimental conditions are standardized for each measurement. In this context, we established a constant timeline in the preparation steps of the samples and we used constant experimental parameters in acquiring the SERS spectra. The used *E. coli* strains are rough (K12)-MG1655, TOP 10 ([Figure 5A](#A) and [C](#C)) and smooth strains (B2)-536, UTI89 ([Figure 5B](#B) and [D](#D)).

**Figure 5.** Raw SERS spectra of rough (A and C) and smooth (B and D) *E. coli* strains collected by using the in situ synthesis approach (Bacteria@ Ag NPs).
Specific SERS bands in each case (with or without the O-antigen) are discussed in our previous study [14], where a clear discrimination between K12 and B2 strains is demonstrated by using chemometrics (principal component analysis, PCA).

In the last decades, SERS was used to identify: DNA bases [58], a wide range of explosives and trace materials [59], food additives [60], therapeutic agents [61], different species of pathogenic and nonpathogenic bacteria [62–64], protozoa [65], fungi [66, 67], and their spores [68], respectively. Furthermore, as previously described, vibrational spectroscopy can be used to study the uniqueness of microorganisms. Consequently, we envision that the in situ synthesis approach could be used to some extent in other microorganisms’ detection as well, not only bacteria.

Particularly, Raman and SERS spectroscopies were already applied in the detection, characterization, and monitoring of growth cycle for fungi. For example, various pathogens such as Candida albicans, C. glabrata, and C. tropicalis isolated from blood cultures have been rapidly identified [66]. Rapid diagnosis of infections caused by fungi from Candida genus is extremely important, since intra-abdominal infections caused by these fungi lead to high mortality rates [67]. Yang and Irudayaraj [69] were able to easily identify A. niger and F. verticillioides pathogenic fungi from apple surface, using FT-Raman spectroscopy. Szeghalmi and coworkers [70] studied the growth of A. nidulans strain A28 hyphae over the Au-coated Klarite SERS substrate, and they detected a strong signal in the close proximity of the hyphal cell wall because of the excretion of some extracellular components during growth.

Another field of interest in fungi studies using Raman spectroscopy and SERS is the characterization of various bioactive compounds extracted from different fungi. De Oliveira and coworkers [71] successfully identified the chemical composition of the extracts obtained from P. sanguineus fungus. The major bioactive components of P. sanguineus extracts are ergosterol and cinnabarin, the last one being responsible for the antibiotic activity of the extract [72].

Zinc oxide nanoparticles (ZnO NPs) were tested for their antifungal activity against B. cinerea and P. expansum fungi. He and coworkers [73] used traditional microbiological plating, along with SEM and Raman spectroscopy and showed that a concentration higher than 3 mmol/l of ZnO NPs can significantly inhibit the growth of B. cinerea and P. expansum. The last one is more sensitive to the action of ZnO NPs because these inhibit the development of conidiophores and conidia, resulting in the death of fungal hyphae. The detection of fungal infection in mice lungs with P. brasiliensis and follow-up treatment with magnetic NPs functionalized with amphotericin B can be achieved using SERS analysis [74].

SERS imaging and analysis have been effectively used for the characterization of in vitro biosynthesis of NPs by different species of fungi. In this regard, Mukherjee and coworkers [75] established a controlled biosynthetic route to obtain the nanocrystalline Ag particles using T. asperellum. Using TEM and XRD, the obtained Ag NPs were found to be in a range of 13–18 nm. C. cladosporioides was also reported to be able of Ag NPs extracellular biosynthesis [76]. In fact, fungi are not only able to biosynthesize Ag NPs, but also Au NPs. Extract from the filamentous fungi A. nidulans was used in the formation of Au NPs within and adjacent to hyphae. Also, the Neurospora crassa extract was tested by Quester and his coworkers [77] for the formation of Au NPs under different experimental conditions. The authors were able to
synthesize Au NPs with different shapes and sizes ranging from 3 to 200 nm by using methylene blue as target molecule.

Concluding this chapter, it is a challenge to entrench how to use most effectively the SERS effect in our favor. The simple reasoning is that SERS is still a not fully understood phenomenon. However, the ongoing studies in the biomedical area show the huge potential of this ultrasensitive technique to actually improve our life quality and the diagnosis procedures of infections and to significantly prevail essential real-life issues. Apart from infections diagnostics, cancer treatment or imaging, drug delivery, and personalized medicine or other health care branches can greatly benefit from Raman/SERS detection and mapping in synergy with functionalized NPs and high-performance support vector machines.

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