

# Surface-enhanced raman spectroscopy (sers)



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Modern biomedicine advances have impelled the demand of sensitive, accurate and fast analytical techniques for biodetection and biondiagnosis. Many tools ranging from fluorescence spectroscopy, mass spectrometry or immunoassays have been used to resolve analytical problems related with health. Although these techniques are well established, several drawbacks still limit their fully applicability, e. g. prior sample preparation, time consumption or relatively low detection limits[1]. Recent developments in laser technology and nanoscience have converted surface-enhanced Raman spectroscopy (SERS) as one of the techniques with highest analytical potential. The SERS effect is associated to the intense electromagnetic field enhancements provided, upon light excitation, by localized surface plasmon resonances (LSPRs) at the surface of a plasmonic metal with features in the nanoscale range [2]. The Raman signal of the molecules in close vicinity to the metal substrate is enormously intensified, allowing sensing applications down to single-molecule [3]. Since its initial discovery, almost 40 years ago, the field of SERS spectroscopy has experienced an exponential growth, renewing the interest within the scientific community[4]. Among numerous potential applications, SERS has emerged as an ultrasensitive tool for detection and identification of biomolecules, such as DNA. [1, 5]. However, although a wide range of indirect SERS-based DNA approaches have been developed (mostly relying on the mediated capture of the target DNA and the use of extrinsic SERS reporters), the direct SERS spectral analysis of unmodified DNA still remains scarcely used, despite the fact that it provides specific, selective and complete vibrational information. The major limitation has been so far the lack of spectral reproducibility at low DNA concentrations. The investigation of the native structure of DNA (especially

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double-stranded DNA) based on its affinity towards gold/silver substrates was mainly hindered by the phosphate backbone of DNA, since it is negatively charged at physiological pH. Recently, the spectral reproducibility issue have been addressed by different scientific groups based on the interaction of DNA with negatively charged nanoparticles, which have renewed the interest indirect SERS-based strategies of detection of DNA.[6-9] On the other hand, Alvarez-Puebla and co-workers developed a successful alternative strategy based on the use of positively-charged silver colloids coated with spermine molecules (AgNP@Sp).[10, 11]

DNA is the carrier of all genetic information in living organisms. Although it is a stable macromolecule, can be subjected to changes such as mutations derived from evolution, genetic diseases or genomic aberrations which can lead to cancer.[12] Apart from the known four canonical bases contained in DNA and RNA, a variety of modified nucleobases play a major role in gene regulation.[13] In higher organisms, epigenetic information for gen regulation is needed in order to determine their functions and properties. 5-methylcytosine is the most investigated modification in mammalian genomic DNA. However, many cytosine variants in DNA regulatory network such 5-formylcytosine, 5-carboxylcytosine and 5-hydroxymethylcytosine have been recently identified. In fact, 5-hydroxymethylcytosine is produced in mammalian tissues and depleted in human cancer cells.[14] These discoveries have suggested that new nucleotide variants possess epigenetic functions for gen regulation that have been underestimated.

In addition to epigenetic modifications, an ample variety of nucleobase lesions (such as alkylation, oxidation, deamination, and cross-linking) can

take place as a result of carcinogen attacks to DNA.[15, 16] In some cases, carcinogens are inherently reactive toward DNA while others require to be firstly metabolically activated to electrophilic intermediates such as phagocyte-generated reactive oxygen, nitrogen and halogen species.[17-19] Anyhow, all these DNA lesions are mutagenic or cytotoxic and, if not properly repaired by the corresponding enzymatic systems, may induce base mispairing during DNA replication.[16] Accumulation of such mutations in genes controlling cell growth, proliferation, programmed cell death, and cell differentiation is likely to cause cancer.[20, 21] Reactive oxygen species (ROS) are possibly the main class of DNA damaging agents, which generates a wide set of different oxidative lesions [22] such as those produced by the direct attack of the highly reactive hydroxyl radical ( $\bullet\text{OH}$ ) to the nucleobase double bonds. On the other hand, inflammation-induced formation of 5-halocytosines adducts (5-chlorocytosine and 5-bromocytosine), have been identified in DNA from human tissues.[23-27] Importantly, 5-halocytosines could act as fraudulent epigenetic signals, in part explaining the link between chronic inflammation and cancer.[28-33]

Due to the recent discoveries of new nucleotide variants with epigenetic functions, an intense research has been directed toward the development of novel methods to detect, profile, and sequence these base modifications in the genome and transcriptome.[34] These strategies span from pure detection and quantification methods (thin layer chromatography (TLC) analysis,[35] antibody-based detection,[36, 37]  $^{32}\text{P}$ -postlabelling[15] and liquid chromatography (LC)-mass spectrometry (MS)[38]) to genome-wide profiling methods and single-base-resolution sequencing methods.[39] A

relatively new field in this exciting area is the screening of DNA samples for unknown or unanticipated lesions, which is referred to as “ adductomics”.

[40] Nowadays, this sort of analysis is primarily performed with liquid chromatography coupled with high-resolution/accurate mass spectrometry (LC–MS<sup>n</sup>). [15, 40] This technique relies on the fragmentation of protonated modified nucleobases which are then differentiated according to their molecular mass. [15, 40] However, DNA adduct analysis by mass spectrometry is costly and time-consuming since it requires a prior sample preparation which normally involves several standard steps, such as DNA hydrolysis into the corresponding monomers, enrichment of the DNA adducts, removal of unmodified nucleobases and addition of an appropriate internal standard. [40] Extreme caution must be paid to avoid artificial generation of DNA lesions during these processing steps.

This research project aims the direct SERS detection of four different cytosine variants in single-stranded DNA sequences, by means of AgNP@Sp colloids. 5-methylcytosine ( <sup>m</sup> C) and 5-hydroxymethylcytosine ( <sup>hm</sup> C) were investigated due to their epigenetic importance in mammalian DNA. 5-bromocytosine ( <sup>Br</sup> C) and 5-hydroxycytosine ( <sup>h</sup> C) were studied as a representative modification involved in DNA damage.

### 1. Transition from Raman to SERS

The Raman phenomenon is related to the process where inelastic scattered photons are simultaneously emitted upon monochromatic light interaction with a specific molecule (Figure 1a). During the scattering process, a large majority of the photons are scattered at the same incident energy (Raighley

scattering), whereas a small part of photons either gain or lose energy (anti-Stokes and Stokes Raman scattering respectively).[41] The resulting Raman spectral bands correspond to vibrational and rotational transitions which are specific to each molecular system providing a vibrational “fingerprint”. Raman scattering is a weak phenomenon since nearly 1 in  $10^6 - 10^{10}$  photons are inelastically scattered[42]. In most of the Raman studies the fraction of the spectrum involving anti-Stokes bands is usually ignored due to the weakness of the bands (Figure 1b). Although the invention of the laser in 1960s implied great improvements in Raman spectroscopy, the lack of sensitivity was still a severe limitation. In 1974, Fleischmann et al.[43] observed for the first time an unexpected dramatic enhancement of the Raman signal from pyridine in presence of rough silver electrodes. Later in 1977, a series of independent reports of Jeanmaire et al.[44], and Albrecht et al.[45] explained the origin of such enhancement, which renewed the interest in Raman techniques and paved the way to Surface Enhanced Raman scattering.

There are two widely accepted theories that explain the origin of SERS enhancement compared to Raman scattering (Figure 1c): the electromagnetic enhancement (EM) and the chemical enhancement (CE). The EM is considered to be the major contribution of the enhancement and is highly influenced by the characteristics of the metal (composition, shape, size). This EM effect arises upon light interaction with a noble metal surface, generating collective oscillations of conduction electrons named as surface plasmon resonances (LSPRs). The coupling of these LSPRs with the emission of the analyte adsorbed or in close vicinity to the metal surface is

responsible for the scattering intensification, which can reach values up to  $10^{10}$  -  $10^{11}$  [41]. In particular, large local electromagnetic fields are observed at the inter-particle junctions (known as “hot spots”) of noble metal nanoparticles which are in close proximity (mostly Ag and Au). Among others, the EM enhancement drastically decreases as the distance between the analyte and the metal surface increases [42] which explains the requirement of close proximity between the nanostructured surface and the target molecule.

The CE contribution to the SERS signal enhancement is usually weaker and, differently to the EM mechanism, is molecule-specific. In fact, when the analyte adsorbs onto the metallic substrate, a new surface-complex is formed and, new electronic transitions may be possible due to the change in the analyte properties such as the Raman polarizability. These transitions are comparable to the Resonant Raman transitions, which analogously lead to higher Raman cross sections. If both mechanisms (EM and CE) are present, their effect is assumed to be multiplicative[46].

Figure 1.(a) Schematic comparison between Raman and SERS effects (b) Raman and SERS spectra of Rhodamine G (c) SERS enhancement mechanisms\*\*\*.

## 1. 2 SERS substrates

For SERS applications, noble metals substrates (mostly silver and gold) must contain features in the nano-range scale (not higher than the excitation wavelength). In fact, whereas large nanostructures highly hamper the LSPRs,

too small nanostructures cannot support these plasmon resonances, leading to low enhancement factors [41].

During the past years great efforts have been made to coherently engineer SERS substrates. Two main approaches have been used for the design of SERS substrates: top down approaches, involving mainly lithographic techniques, and bottom up approaches.[47] Top down approaches allow the controlled manipulation of interparticle gaps and relatively simple functionalization of metal surfaces. In the case of bottom up approaches, nanoparticles are chemically synthesized and subsequently assembled in suspension or in ordered manner.

In this context, silver and gold nanoparticles are the most used SERS-active substrates due to their outstanding optical response. Noteworthy, the LSPRs of silver substrates cover a wider wavelength range (comprising most of the visible and nearinfrared spectral region) than gold substrates. Below 600 nm, gold nanoparticles mostly absorb the incident light, hampering the surface plasmon resonances in this spectral range. [48] On the other hand, gold nanoparticles are more stable under oxidative conditions, easily prepared and with high potential tunable plasmon properties. [49]

### 1. 3 SERS instrumentation

Current dispersive Raman instruments are coupled with microscopes and several laser beam lines as excitation sources. The choice of the excitation wavelength (mainly from the visible to NIR) depends strictly on the application. An important factor to take into account when performing a SERS measurement, is the Raman scattering efficiency since it depends on

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the fourth power of the frequency[50]. Therefore, shorter wavelengths improve the Raman sensitivity although the risk of fluorescence or sample degradation also increases and has to be considered [51].

In this particular set-up and in most modern Raman spectrophotometers, the inelastically scattered light is collected at  $180^\circ$  geometry. In the confocal unit, the light from the laser initially passes through a pinhole aperture. This light is then delivered through the notch filters (interference filters) where it is completely reflected into the microscope and reaches the sample through the beam splitter. Then, the inelastically scattered light passes back through the same optics, a monochromator and finally reaches the CCD detector. The Rayleigh scattering is efficiently blocked by the notch filters.