

Common Aspects Influencing the Translocation of SERS to Biomedicine



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ARTICLE HISTORY

Received: July 27, 2017

Revised: December 05, 2017

Accepted: December 05, 2017

DOI:

10.2174/0929867325666180105101841

Abstract: This review overviews the impact in biomedicine of surface enhanced Raman scattering motivated by the great potential we believe this technique has. We present the advantages and limitations of this technique relevant to bioanalysis *in vitro* and *in vivo* and how this technique goes beyond the state of the art of traditional analytical, labelling and healthcare diagnostic technologies.

Keywords: Biomedicine, bioanalytes, Surface-enhanced Raman Scattering (SERS), nanoparticles, hybrid plasmonic platforms, biosensors, multiplexed bioanalysis, early diagnosis.

1. INTRODUCTION

Modern bioanalytical assays require highly specific and ultra-trace level quantitative analysis for rapid and reliable examination of biological phenomena. Several well-established and commercially available analytical methods fluorescence, liquid/gas chromatography, atomic absorption or emission spectroscopies, mass spectrometry, or capillary electrophoresis show high selectivity and sensitivity down to ultra-low levels of detection [1]. However, they all present significant limitations. For example, all techniques require extensive sample manipulation prior to analysis, and some, such as liquid/gas chromatography and atomic absorption or emission spectroscopies, are destructive. Furthermore, capillary electrophoresis presents low sensitivity. The high operating temperatures in gas chromatography are restrictive for the accurate quantification of thermally labile moieties [2]. In this respect, high-performance liquid chromatography offers a useful alternative to gas chromatography methods, however, only molecules with good absorbance in the UV-vis region can be detected. Fluorescence has evolved tremendously over the years due to technological advances in the instrumentation, and the availability of a

large collection of more efficient and tuneable fluorescent probes such as organic dyes and inorganic nanoparticles (NPs) [3]. While this method offers reliable, non-destructive, rapid, and sensitive detection for a wide range of biomedical applications, it is prone to photobleaching and severely counterfeited by the intrinsic autofluorescence emissions of biological matter [4]. Besides, fluorescence is inefficient in cases where multiplex detection is required due to the broadness and overlap of the fluorescence peaks [5] phototoxicity issues and impairment of subcellular components may arise upon illumination for example due to the production of reactive oxygen species (ROS) [6].

Other strategies for quantitative analysis such as enzyme-linked immune-sorbent assay (ELISA), typically used to test blood glucose levels, HIV, and entero-toxin [7], polymerase chain reaction (PCR), and multiplex ligation-dependent probe amplification techniques, employed in disease diagnosis, genotyping, pathogenic environmental monitoring, public safety, and healthcare screening and testing, are expensive and time consuming. ELISA assays may also produce false positives and inaccurate identification of toxic species. Specifically, inaccurate readout of amyloid- β (A β), the main toxic species related to Alzheimer's disease, occurs as other antagonistic ligands cross-bind and interfere with the assay antibodies [8].

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In the quest for improved analytical tools, surface-enhanced Raman scattering (SERS) spectroscopy has been recently arisen as a candidate technology to complement and even go beyond the state of the art of traditional analytical, labelling and healthcare diagnostic technologies [9].

SERS takes advantage of the coherent oscillation of the conduction electrons in noble metal NPs and the excitation of localized surface plasmon resonances (LSPRs) at specific wavelengths of incident light. This optical phenomenon induces gigantic electromagnetic field amplification onto the surface of the nanostructure [10]. This enhancement of the Raman signal reaches maximal levels at specific sites, hot-spots, which permit ultra-high sensitivity, even down to the single molecule regime, with ultra-high spatial resolution even within cells. Ultra-trace level analysis is comparable or below other conventional technologies [11]. The lack of water interference allows *in situ* analysis [12]. Besides, the existence of unique Raman fingerprint-like spectral patterns for most chemical and biochemical samples, enables label-free analysis and the simultaneous co-targeting of multiple analytes.

Even though UV-vis-NIR and fluorescence have been widely used in molecular multiplexing, these techniques are limited to two or three targets at a time as they provide broad spectroscopic signals that lack fine structure and precise information [13]. The characteristic SERS spectral patterns with narrow spectral features carry unique molecular information acting like vibrational barcodes even at the level of chemical moieties, allowing multi-analyte detection.

In biolabelling, Mirkin and co-workers [14], as well as Moskovits and co-workers [15], reported that SERS-based biotags can be more efficient than conventional fluorescence tags for biological imaging and detection [16]. In healthcare diagnosis, remote and high-throughput screening of multiple samples was also accomplished by SERS using specially designed microfluidic devices, allowing on-chip detection with nM range sensitivity [17]. Authors of this study found the SERS cross section to be 3.3 times greater than the fluorescence one over the same wavelength range, enabling their routinely use in applications such as flow cytometry. In addition, the nanometer-sized SERS platforms permit multi-degree miniaturization and application even in living cells [18]. Little or no sample manipulation before SERS examination allows fast assays at minimal cost.

Remarkably, the main limitation in SERS stems from the same source that generates the Raman en-

hancement, the plasmonic substrate itself. Inconsistent signal intensities and reproducibility issues derived by the SERS-active substrates impeded until now SERS spectroscopy to be implemented in routine bioanalysis. The answer for the ideal SERS platform is not simple, as it does not only depend on the fabrication of sophisticated and rationally designed nano-objects. It also depends on controlling the biointerface that can actively interact with the biotargets. Along this review, we will comment these two aspects.

2. PARAMETERS INFLUENCING SERS BIOANALYSIS

2.1. SERS Active Platforms

From the SERS platform point of view, the fundamental metric for SERS activity is the enhancement factor (EF), which quantifies the increase in signal intensity ($\text{counts s}^{-1} \text{mW}^{-1}$) per molecule. Most SERS substrates have EFs in the range 10^3 – 10^8 . EF depends on several parameters of the SERS platform, including geometry and composition [19].

Noble metal colloids in suspension or immobilized in solid templates (glass, silicon wafer, organic or inorganic colloids) are, by far, the most used SERS-active substrates as their LSPRs can be easily excited in the vis or NIR regions [20]. Isolated particles, spherical-shaped colloids, mostly made of gold (Au) and silver (Ag), produce moderate EFs on the order of 10^2 – 10^3 . Other shapes such as nanocubes, star-shaped NPs, nanotriangles, and in general particles exhibiting sharp edges and tips, yield higher EFs up to 10^7 . Narrow gaps (<1 nm to a few nm) formed in fractal agglomerates of plasmonic NPs for example, host optical hot spots. The electric field is confined in such nanogaps generating SERS EFs $>10^6$. Such random particle assemblies lack of reproducibility of the location, and density of hot spots. Various fabricating methods are employed to reduce the randomness of hot spots and limit the SERS intensity irreproducibility as described below.

Colloids can be adsorbed onto solid supports to create SERS-active substrates. To control their adhesion, several strategies are employed. In the self-assembly of NPs (the primary building blocks) onto the substrate [21], the driven forces are electrostatic interactions, capillary force deposition, and *in situ* chemical or photochemical particle growth. Nanolithography methods result in a patterned substrate with a high degree of control over material parameters and characterized by high EFs [22]. These methods include electron-beam lithography consisting in chemical etching, NPs array “printing” using chemically modified polymers, or

nanohole arrays by focused ion beam (FIB) technique. Khlebtsov *et al.* propose Au nanoisland films (NIFs) as a highly sensitive and reproducible SERS platform for fungicide detection [20a]. The calculated EFs of the NIFs compared to Au nanostars and Ag nanocubes assemblies, found to be approximately one order of magnitude higher indicating the robustness of such SERS platform reaching thiram detection as low as 4.8 ng/cm².

Alternative to Au and Ag materials, metal oxides and semiconductor materials have also been proposed as SERS platforms. Advantages of oxide-based nanostructures such as Fe₃O₄, TiO₂, WO₃, ZnO or TiO₂ include cost efficiency, ease modification, biocompatibility, and some bring new properties to the material. In the case of metal oxide nanoparticle-enhanced Raman scattering (MONERS), the reported enhancements obtained on flat metallic surfaces are typically 3-4 orders of magnitude. This is comparable to Raman enhancements on gold spheres. Additionally they can be excited in a wider range of the spectrum [23].

A metal-free SERS substrate by employing non-stoichiometric tungsten oxide W₁₈O₄₉, shows SERS EF as high as 3.4×10^5 and 100–10,000 times higher than the previously reported values for most other semiconductor SERS-active substrates and comparable to noble metals [24].

3D zinc oxide tetrapods (ZnOTP) decorated with branched AuNPs nucleated and grown in situ present outstanding SERS signal enhancement with a calculated EF close to 10^7 . With this material 1 μM of apomorphine, a Parkinson disease drug, could be quantified at different drug-loaded solutions [25].

Li *et al.* suggests copper-based chalcogenides as SERS substrates. This material is a p-type semiconductor due to the presence of copper vacancies. Highly monodisperse CuTe nanocubes, nanoplates, and nanorods showed strong plasmonic band at 900 nm [26]. Nanocubes appeared to be more sensitive toward Nile red even when compared to Au nanostars due to low affinity of ketones for Au suggesting their utilization for oxygen-substituted targets [26].

Nanoscaled transition-metal chalcogenides (TMCs) show interesting properties arising from the synergetic effects between the component materials found in the heterostructures formed by a combination of metal NPs with semiconductors. In particular, Cu₂Se/Pd heterostructures show greater SERS performance than pure Pd or Cu₂Se revealing phase- and composition-dependent characteristics [27].

The semiconductor material can be used directly as a substrate to enhance Raman scattering, a process called semiconductor enhanced Raman scattering. It can also act like an “antenna” or “trap” to modulate the Raman enhancements originating from the metal/semiconductor heterostructure, a process called semiconductor-mediated-enhanced Raman scattering [28]. Here, the Raman enhancement stems from a variety of contributions, including surface plasmons, charge transfer, molecular, and exciton resonances. Even though the theoretical background of this technique is still evolving, the overall EF is expected to be about 10^6 from plasmon resonance originating from the valence band of the semiconductor, plus a 10^3 -enhancement due to charge transfer resonances between the molecule and the semiconductor, and finally an additional enhancement to the Raman signal of the order of 10^6 , originated from Mie resonances found in such high refractive index materials. Overall reaching outstanding and previously unseen levels of EF enhancement.

Wong and co-workers have recently developed a novel platform inspired by nature, the Asian pitcher plant. It enables the delivery of target molecules originating from various phases to the SERS substrates [29]. Slippery liquid-infused porous surface-enhanced Raman scattering (SLIPSERS), with EF comparable to conventional particle agglomerates, combine the desirable high specificity along with single molecule sensitivity in highly diluted analyte solutions.

2.2. The Nanomaterial-biological System Interface

SERS-active biointerface engineering is the key for an efficient bioanalysis especially at low concentrations [29, 30]. The complex composition of most bioanalytes (including cells or bacteria) together with their large sizes, and in many cases the lack of specific chemical groups with affinity to the plasmonic surface led to poor SERS performance in terms of both selectivity and sensitivity. Big biological systems have been reported to show inconsistent, inhomogeneous, and position dependent SERS signals. Thus, the linear response between their concentration and SERS intensities is inhibited and quantitative analysis hampered [20b].

Rationally designed nanostructured porous plasmonic substrates enables *in situ*, label-free detection of quorum-sensing-regulated, low-molecular-weight microbial metabolites [31]. Furthermore, metabolites produced after interspecies chemical communication could be identified in cocultures onto agar-based hybrid plasmonic platform [32].

Complex biological media may also put in question the ultra-sensitivity and the molecular specificity of SERS, due to background noise of interfering species and/or nonspecific adsorption of proteins impeding the target analytes to reach the metal surface.

On one hand the shape, topography, surface properties and even the elastic modulus of the nanomaterial affects the adsorption kinetics of the biomolecule. On the other hand, the enhancement strongly depends on the distance, and the large size of biomolecules promotes the Raman signature of the molecule be differently enhanced depending on the biomolecule conformation onto the substrate.

SERS-active substrates optimally adapted in shape and spacing, have demonstrated improved sensitivity. For example, viruses of different sizes, including adenovirus (Adeno), encephalomyocarditis virus (EMCV), and influenza virus with its three strains (H_1N_1 , H_2N_2 , and H_3N_2) were successfully detected by precisely fabricated hexagon-like Au nanorod arrays with a controlled size and spacing to match the needs of each target [33].

In order to unmask and promote only the desired analyte signals, Sun *et al.*, reported accurate fructose SERS determination in clinically relevant concentrations, in the mM range and in complex protein solutions, by introducing a customized “silent layer” with very weak Raman cross section, to filter and leave unperturbed signals arising only from the fructose probe [34].

3. BIOACTIVE ANALYTES DETECTION METHODS

3.1. Direct Detection

A straightforward approach in SERS studies relies in the direct contact of the desired analyte with the bare plasmonic platform, being itself the sensing element for a single or a few targets [35]. When the number of targets under direct simultaneous examination is increasing and multiplex detection is aimed, the resulting information should permit thorough differentiation of analytes. Chemisorption or at least a very close distance of only few nanometers between the analytes and the plasmonic surface is indispensable [36]. The most common methods can either consist of modifying the surface charge of the SERS substrate to enable electrostatic attraction of targeted molecules, or creating a layer with trapping sites for analytes with low Raman cross-section [37]. While SERS methodologies are ad-

vantageous in many aspects, these direct approaches are hampered by the complexity of the sample, the number of targets under study in each assay, the background components present in the media running the experiments, and the analyte's affinity towards the SERS platform predominantly made of Au and Ag. Again, vibrational mode overlapping restricts multiplex direct SERS detection when a high number of targets coexist with background components that may be antagonistic to each other.

Nevertheless, SERS is versatile enough to allow developing strategies to overcome the reduction in selectivity and sensitivity that direct methods show.

Recognition of non-anticipated lesions when screening DNA samples, known as adductomics, is possible through a combination of chemometrics, microfluidics and direct SERS, capable to accurately identify and quantify chemical modifications in canonical nucleobases of both nucleic acids for rapid, low-cost and high-throughput screening analytical devices [38]. Intrinsic SERS investigation of unmodified DNA is mainly restricted to ssDNA, as the direct contact of dsDNA with the nanostructured metallic surfaces is hindered by the negative charge of the phosphate backbone resulting in poor sensitivity and spectral irreproducibility. A label-free SERS alternative for the study of unmodified duplexes, dsDNA, is based in the fabrication of positively charged Ag NPs coated with spermine molecules (AgNP@Sp) [39]. Spermine molecules stabilize the AgNPs, and upon addition of negatively charged DNA, it promotes controllable NPs aggregation into stable particle clusters (Fig. 1) with no need of external aggregating agents. The DNA-AgNP@Sp conjugate is formed by non-specific electrostatic interaction of the phosphate groups rather by base-specific nucleoside-metal binding. Thus, a sandwiched configuration is formed between dsDNA at the interparticle junctions, the so-called electromagnetic hot spots, maximizing the intensification of the Raman signal reaching nanogram sensitivity.

In case of more complex targets, SERS recognition, *in situ* analysis, and monitoring in cells is possible by examining the secreted non-specific chemicals during their growth, metabolism, proliferation and apoptosis [40]. Thus, SERS investigation of the presence of certain chemicals and/or proteins around isolated or grouped cells permits profound understanding of their overall functionalities and communications in case of neighbouring cells and even live or dead cell discrimination.

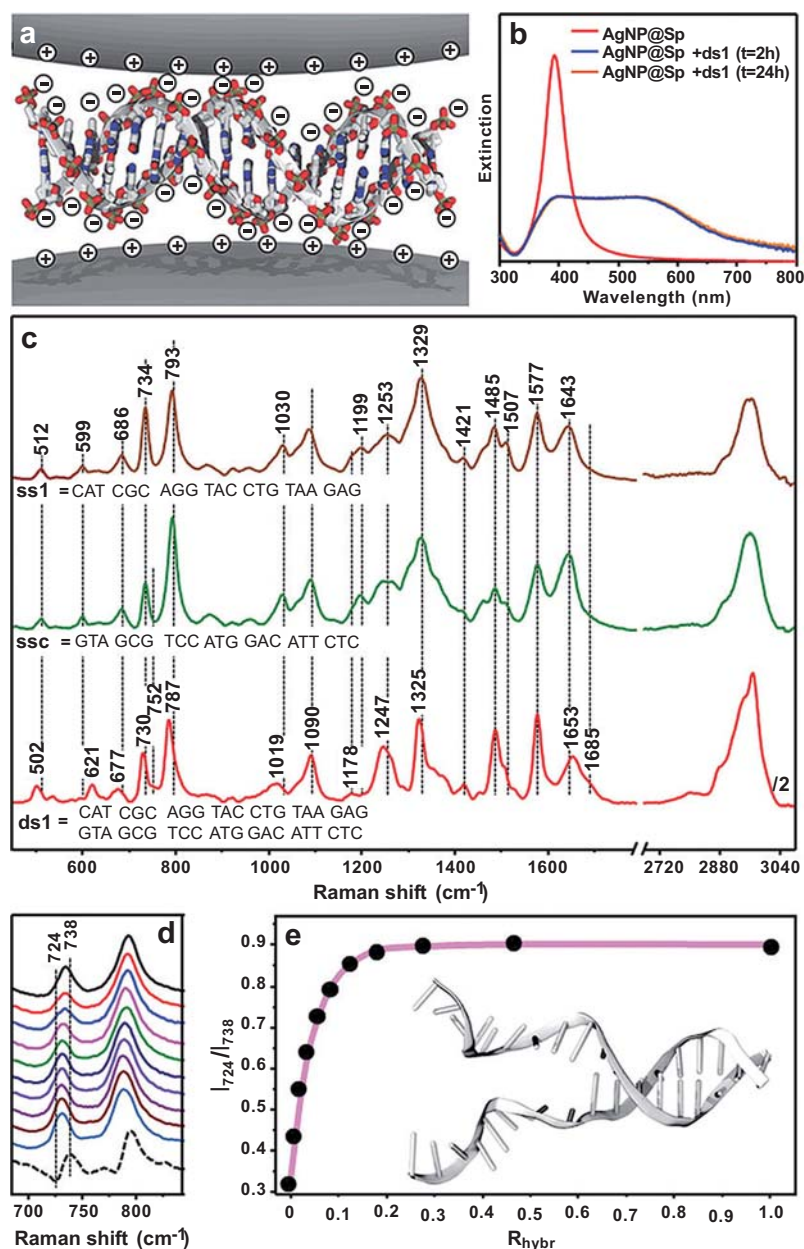


Fig. (1). SERS as an alternative to PCR to detect double stranded DNA, to quantify hybridization events and DNA alterations. (a) Scheme of double stranded DNA (dsDNA) sandwiched between two positively charged silver nanoparticles conjugated to spermine (AgNP@Sp) (grey areas). (b) Extinction profile of the free nanoparticles (red curve) and nanoparticles with agglomerated DNA onto their surfaces after adding DNA at 2h (blue curve) and 24h (orange curve). (c) SERS profile of the single stranded DNA (brown spectrum), its complementary sequence (green spectrum) and the double stranded DNA (red spectrum). (d) SERS spectra of single stranded and double stranded DNA mixtures (ssc:ds1) at increasing molar ratios (R_{hybr}) from top to bottom showing a shift of the adenine band from 734 cm^{-1} to 730 cm^{-1} . The dotted spectrum represents the difference spectrum (ssc-ds1) from (c) showing a minimum (724 cm^{-1}) and a maximum (738 cm^{-1}). (e) Ratiometric measurements of these two bands vs. the molar ratios (R_{hybr}) allows identification of double and complementary strand DNA populations. (Reprinted with permission from ref. [39]).

3.2. Indirect Detection

SERS is proven to be useful to many biosensing applications in virtue of its ability to engage different approaches of analyte targeting. Complementary biodection methods involve indirect intrinsic or extrinsic

SERS strategies. Depending on whether the recorded spectral information is attributed to the vibrational pattern of a ligand with the ability to react with high specificity in presence of certain bioanalytes, or to a Raman reporter molecule generating the signal for detection, thus acting as cellular or molecular tag. Such detection

schemes are considered indirect because the signal originates from the label or the specific receptor-ligand molecular recognition event such as antibody-antigen, and not from the analyte itself, enabling high-throughput screening, multiplexing, and greater versatility for sample analysis within complex biological fluids such as saliva, blood, urine, or sweat.

Among the SERS biodetectors, sensors sensitive to ionic species or small molecules as endogenous bioactive gases (NO) [1, 41] concentrate a lot of interest as they play an important role in cellular function. Abnormalities in their content in human physiology may be linked to a great number of human diseases and for this reason SERS sensing strategies in this direction are investigated more extensively nowadays. This increased interest towards selective monitoring and quantification of monoatomic and/or molecular ionic species, related to the physiological or disrupted cellular function, is important to clinical diagnosis and human health assessment.

Monoatomic ions lack vibrational signatures disabling SERS analysis. A strategy is to monitor the SERS vibrational pattern changes that undergoes a spe-

cially designed ligand in response to the ion presence. Ion sensitive SERS nanosensors have been reported in many cases as H^+ (pH) sensing based on the ionization/deionization of the carboxylic group of 4-mercaptobenzoic acid (MBA) [42], halide sensing as in the case of Cl^- [43] upon interaction with a Cl^- -sensitive molecular probe that has a high SERS cross-section, Hg^{2+} [44] based on its coordination with human telomeric G-quadruplex DNA, Cd^{2+} [45] using Raman-encoded NPs, alkaline and alkaline earth metal cations by electrostatic interactions [46].

A label-free indirect ultradetection of relevant bioanalytes is demonstrated by SERS through coupling a specific monoclonal antibody (mAb) directed against benzoylecgonine (BCG), a major cocaine metabolite expressed in biological fluids such as saliva or blood, as an indicator of drug abuse [47]. For this, an optically stable SERS substrate comprising Ag-coated carbon nanotubes (CNT@Ag), with activity in a wide window of excitation wavelengths, ranging from the visible to the near-infrared (NIR) is developed. Figure (2) gives a schematic representation of this sensor and the SERS spectra of the direct and the label-free specific indirect

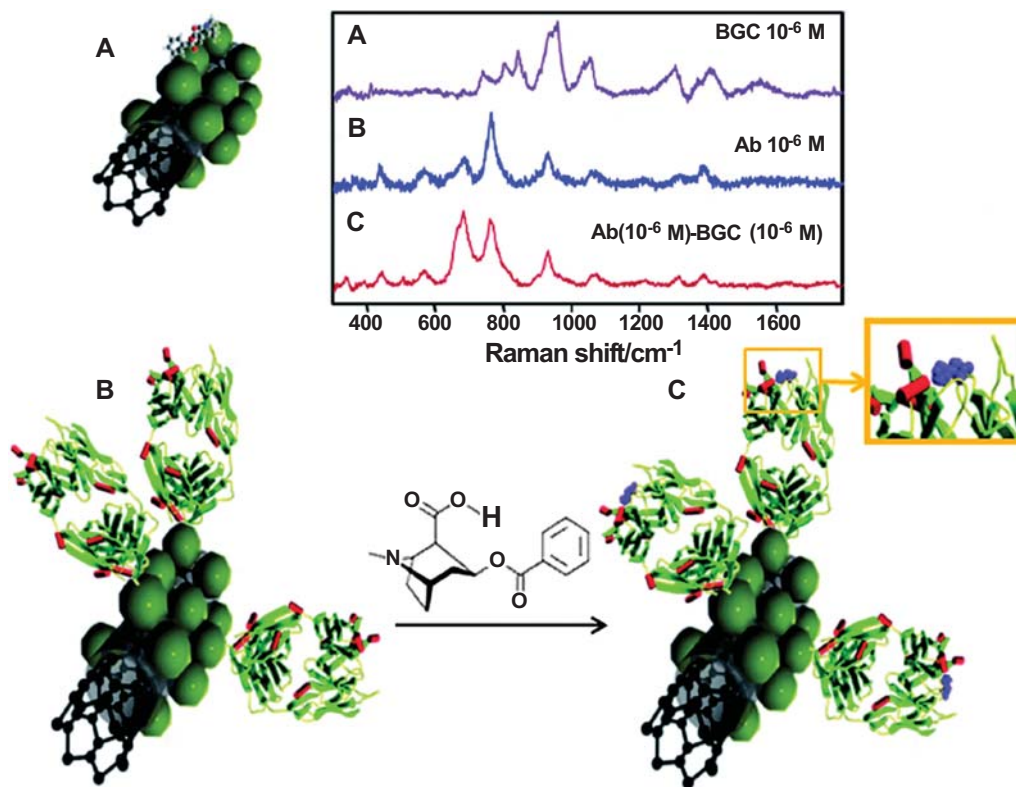


Fig. (2). Cocaine consumption detection system based on carbon nanotubes. Scheme of the SERS sensor consisting of carbon nanotubes (CNT, grey tube) decorated with silver nanoparticles (green spheres) functionalized with the capturing molecule (benzoylecgonine (BGC)) for direct detection of cocaine metabolite (A) or with antibodies (Ab) against the metabolite (B). On the upper right side of the figure, the SERS spectra of the cocaine metabolite (BGC, purple line), the free antibody (Ab, blue line) and of the antibody after detecting the metabolite (Ab-BGC, red line). (Reprinted with permission from ref. [47]).

detection of BCG on CNT@Ag. Comparison of the quantification efficiency of the proposed label-free indirect SERS detection of BCG against direct or various other methods range in the same nM range.

Encoded platforms, or other hybrid particles are used in SERS in a similar manner as fluorophores are used in immunology bioassays. The use of bioconjugated encoded platforms for direct use in suspension or for engineering biodetection devices is advantageous due to the unlimited number of different labels, equivalent to barcodes of each entity, while protecting from background signal interference.

Circulating cancer cells could be identified in whole blood with a limit of detection less than 10 cells/ml [48]. Cells were treated with magnetic beads and with antibody-conjugated SERS tags. The magnetic field allows accumulation of the cells in a certain position where the Raman spectrum is acquired after NIR excitation of the Nanoplex biotags [48].

Pathogenic viral antigen detection, as for example Hepatitis B virus in human blood plasma (HbsAg) and Human Influenza B virus (FluB) using microfluidic technologies or lateral-flow immunochromatographic assay (LF-ICA), with SERS immunoassays is based on

Raman-tagged nanostructures with concurrent antibody functionalization, formatting a sandwich immunocomplex formation with the viral antigen [49].

Functionalization of sub-micron beads onto substrates enables colloidal stability when organic ligands with low polarity are assembled over the nanoparticle's surface. This is especially important because they suffer from uncontrolled agglomeration in dispersions, thus hindering the accessibility of the targets with the sensing nanoparticle interlayer. While polystyrene is a cheap substrate option, its interfering Raman emission peaks and high background fluorescence obstructs analyte signals. However, rationally designed SERS nanoparticle probes can overcome this limitation and produce strong immunoassay signals without interference from the polystyrene microplate substrate with assay sensitivity in the low pM range [50]. Hollow heterostructures have also been proposed for SERS encoded detection suggesting an interesting category of miniaturized sensors for biosensing applications with improved multiplexing capabilities. Submicron-sized SERS reactors composed of hollow silica capsules with a high density of metallic NPs decorating their inner surfaces have also been presented [51]. Figure (3) shows their fabrication, codification, biofunctionaliza-

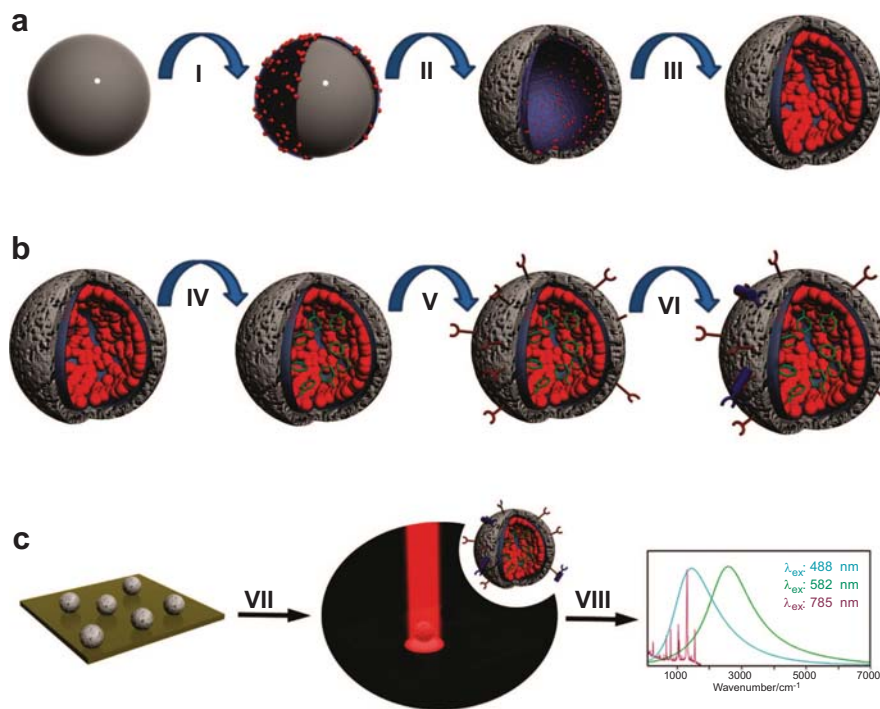


Fig. (3). Synthesis of codified plasmonic nanoreactors functionalized with antibodies for antigen recognition: (a) synthesis of the plasmonic hollow nanoreactors, (b) SERS codification (green rings) of the plasmonic area (red cavity) and surface functionalization with antibodies showing antigen detection (brown-blue complexes), (c) nanoreactors deposited onto substrates (VII) for SERS analysis at 785 nm of the codes (VIII, purple spectrum) and immunodetection based on the colocalization of antibody (Ex. 532 nm, green emission spectrum) and antigen (Ex. 488 nm, blue emission spectrum). (Reprinted with permission from ref. [51]).

tion, and biosensing proof of concept. These capsules allow inner side codification with SERS molecular probes while permitting biofunctionalization of their outer silica surfaces, thus avoiding side effects such as catalytic reactions outside the capsules or optical contamination of the SERS code.

3.3. Multiplexed Bioanalysis

Key for multiplexing is the analyte specific ultra-sensitive fingerprint-like information provided by SERS. Although SERS methodologies have a proven capacity to resolve analytes within complex media [20d, 52], multi-analyte systems are still challenging especially when both sample identification and quantification is intended. One should evaluate several factors when designing multiplex experiments using for example reporter molecules. These include similarities in their molecular structures, differences in their surface adsorption characteristics, absorption maxima of the analyte at the laser excitation wavelength, and the

SERS cross-section of each reporter [53]. Ideally, a reliable platform with comparable signal intensities from the different reporters is needed. Chemometric methods should be employed to resolve the responses from different labels which are hard to distinguish as the number of targets increases.

Scherman and co-workers achieved quantitative SERS-based multiplexed detection of 3 monoamine neurotransmitters in human urine employing chemometric analysis [53]. Exploiting the non-specific supramolecular interactions of a macrocyclic host molecule such as cucurbit[n]urils (CB[n]), enables targeting of a class of molecules over specific analytes. The reproducible SERS signal intensities arise from the precisely spaced sub-nanometre gaps in between neighbouring NPs. SERS data are collected for training predictive numerical models used as a guide for the identification of unknown samples, thus suggesting the applicability of this sensor at clinically relevant levels (Fig. 4).

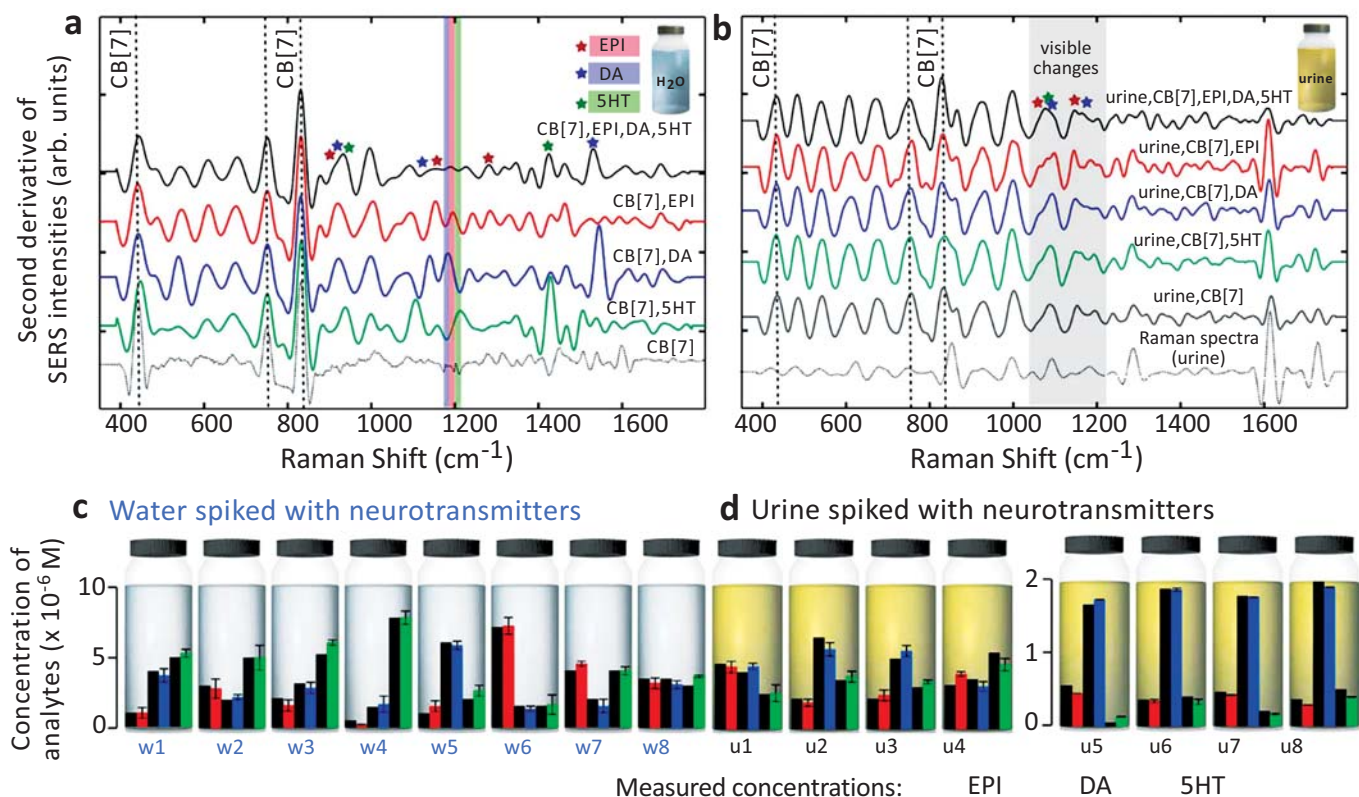


Fig. (4). Example of implementation of numerical chemometric methods in SERS multiplex sensing of neurotransmitters in urine using supramolecular cucurbit[n]urils (CB[7])-gold nanoparticles. Validation in water (a) and in urine (b): for this the SERS profile of the capturing molecule (CB[7]) was recorded alone (grey spectrum) and after its interaction with each neurotransmitter EPI (red spectrum), DA (blue spectrum), 5HT (green spectrum) and a mixture of all (black spectrum). The spectral changes of the CB[7] upon interaction with neurotransmitters are highlighted and indicated with coloured stars. (c-d) Partial Least Squares Regression (PLSR) method is used to predict concentrations of the three neurotransmitters in water and urine media. The last four bottles represent clinically relevant concentrations of the neurotransmitters. Abbreviations: epinephrine (EPI), dopamine (DA) and serotonin (5HT) (Reprinted with permission from ref. [53]).

A quantitative 3-plex sensor multiplexing toxic metal ions, Hg^{2+} , Ag^+ , and Pb^{2+} , down to the nM-pM regimes is based on an alignment-addressed Au nanowires (NWs)-on-chip SERS system using metal ion-specific dsDNAs [54]. The single step detection mechanism relies on the specific binding between the metal ion to its corresponding aptamer, leading to elimination of a Raman reporter-attached DNA from the NW by conformational change of the aptamer. SERS signal crosstalk is avoided by using numerous kinds of aptamers for reliable quantitative real-time and remote monitoring analysis.

3.4. Quantitative Detection

As already stated above, quantitative SERS measurements is key for biological sensing, biochemistry and molecular biology analysis, for the detection of biological warfare agents or pollutants, in forensics science, and for cancer and other disease diagnostics. Numerous research works focus on optimizing the

quantification capacity of the SERS sensors by investigating the influence of uniform and large SERS substrates. It is well known that nanoscale surface morphology of the enhancing surfaces highly impacts the attained sensitivity and detection limits in SERS experiments. The SERS platform should be carefully and rationally fabricated to boost the Raman scattering efficiency. Bottom-up approaches based on the rational design of plasmonic substrates or top-down approaches based on patterned surfaces and nanoscale precision produce robust SERS sensing devices, minimizing intensity signal fluctuations triggered by structural irregularities.

Gwo and co-workers described an example of single-molecule SERS detection using large-scale hot spot engineering [55]. Figure (5) shows two-dimensional hexagonal close-packed superlattices of AgNPs formed with excellent uniformity allowing sensitive SERS detection over a large area.

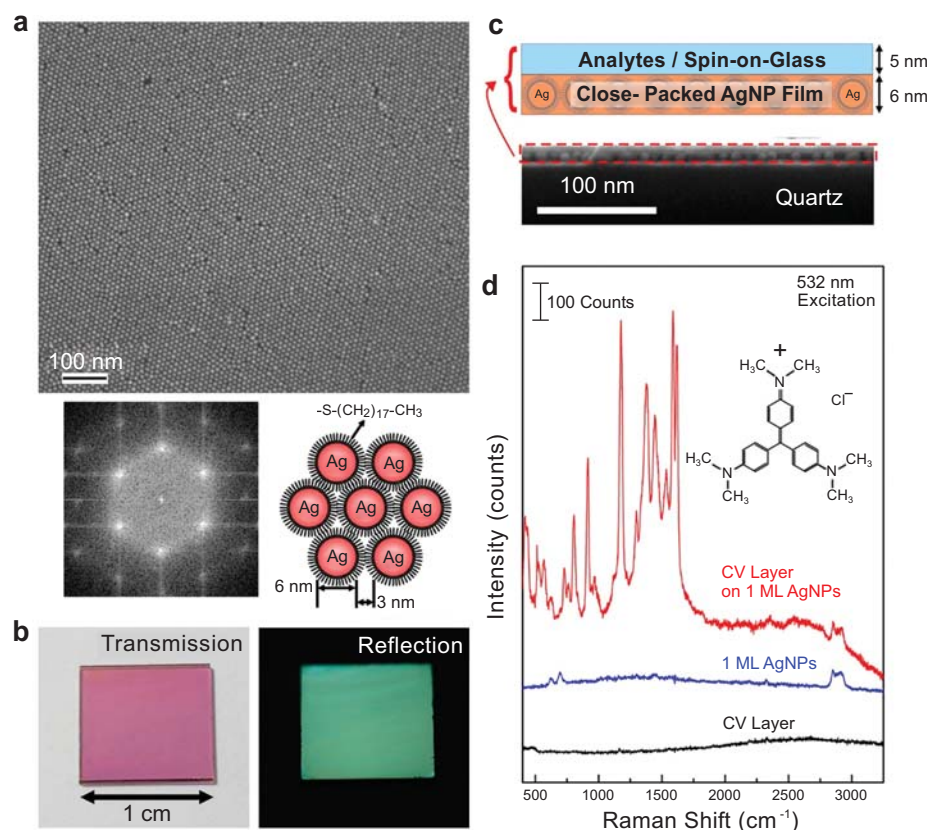


Fig. (5). Highly uniform close-packed silver nanoparticles (AgNPs) superlattices allow single-molecule SERS detection of crystal violet (CV). **(a)** Field-emission scanning electron microscopy image and fast Fourier transform of a single monolayer (1 ML) densely-packed AgNP film with the thiolate chain length defining the interparticle gap (scheme). **(b)** Macroscopic uniform AgNP film showing the difference in colours when the light is transmitted or reflected, because of their optical properties. **(c)** SERS substrate cross-sections indicating each layer. **(d)** Raman spectra acquired from the CV layer on the AgNP film (red spectrum), the bare AgNP film (blue spectrum), and the bare CV layer (black spectrum), respectively at 532 nm excitation. (Reprinted with permission from ref. [55]).

SERS-microfluidic platforms are used for reproducible quantitative analysis, as the continuous flow generated inside the microfluidic devices produces averaged scattering signals. Microfluidic-SERS devices are excellent candidates for the fabrication of Lab-on-Chip systems in the field of bioanalytics. In this context, advances in experimental design, equipment setup and other detection schemes have contributed towards significant improvements in quantitative SERS analysis [56]. Internal standards, alternative sampling methods by collecting multiple points or new protocols for sample preparation are becoming an important tool to resolve variations in the signal intensity in quantitative SERS analysis.

4. DETECTION OF BIOACTIVE ANALYTES *IN VIVO* AND *IN VITRO*

Clear insight over dynamic or regulated processes and molecular events underlying both normal biological functions and pathological abnormalities, is crucial for cellular activity investigation and early disease diagnosis or disease progression in clinical tests. Direct interrogation of biological activity through *in situ* continuous monitoring methodologies in cell cultures, *in vitro* sensing, or even in living organisms, *in vivo* analysis, illustrates in entirety the complexity of these systems. The ideal disease testing strategy for clinical assessment should meet various essential requirements as to be safe, non-invasive, cheap, and standardized, providing precise, reproducible and repeatable results over time across different measurement systems and laboratories.

SERS-based *in vitro* assays and especially *in vivo* diagnostics, come as a potent tool to readily track all the biochemical signals implicated in extra-, intra-, and intercellular communications, without cells fixation or lysis from substrates, as other biochemical and immunochemical techniques often require. The great number of scientific reports in disease diagnosis reflects the potential of the SERS methodologies. Raman-encoded molecular imaging (REMI) technology permits multi-targeting and imaging of cell-surface protein cancer biomarkers on fresh tissue specimens [57], sialic acid (SA)-imprinted SERS nanotags for SERS imaging of cancer cells and tissues [58], SERS tags for multiplexed imaging in living mice [59] or silica-encapsulated SERS NPs that act as a molecular imaging agent to visualize liver malignancies in mice [60].

Regardless the advantages of this technique, there are concerns on the toxicity of some SERS active substrates in addition to the challenge that must be over-

come when accessing the sample in living organisms. In particular, SERS platforms composed of conventional noble metal NPs due to the nature of their material, size, and reactivity with biological molecules such as proteins need to be treated with nontoxic coatings or other biocompatible materials in order to minimize the possibility of an immune reaction from the host [61]. Overall, sophisticated SERS biosensing platforms present controlled bioreactivity, photostability under experimental conditions, multiplex capability, biocompatibility and much research effort is invested in order to achieve negligible side effects on the host organism.

Nano-scale objects composed of Au, varied in size and morphology, are among the most widely used substrates in biomedical applications for their convenient optical properties and biocompatibility. To the latter adds up the fact that can be easily surface modified and coupled with the desired biomolecules such as drugs, proteins, or nucleic acids [62]. Consequently, Au NPs have been extensively used to examine complicated nanomaterial-cell interactions and to elucidate their location within the cells. Cyanine dye (Cy5) labelled Au nanostars have been used to investigate the internalization of such nanomaterials into cells based on the selective quenching of the SERS signals from extracellular NPs through a reaction with tris(2-carboxyethyl) phosphine (TCEP) [63].

Hypertension, atherosclerosis, as well as other inflammatory conditions, are among the vascular diseases that concentrate increased attention. Interestingly, SERS methodologies based on tagged NPs with spectroscopic markers come as a novel tool offering improved insight of the dynamic vascular biology of the endothelium processes and pathological conditions [64]. Nitric oxide (NO) apart from having an important function as an endothelium-derived relaxing factor in vessels, is also an important physiological messenger and effector in mammalian cells. Quantitative SERS analysis based on chemoselective interactions enables *in vitro* remote monitoring of intracellular NO through its capacity to promote diazotization of certain aromatic amines [41]. The ability of the sensor to detect the dynamic intracellular concentration of lysosomal NO, in 3T3 embryonic fibroblasts, can be seen in Fig. (6). SERS methodologies of this type contribute toward intracellular reporters for multiplexed on-line detection of multiple analytes and remote sensing.

Long and co-workers first reported and demonstrated the selective detection of intracellular carbon monoxide (CO) under physiological conditions [65]. Rationally designed AuNPs assembled with palladacy-

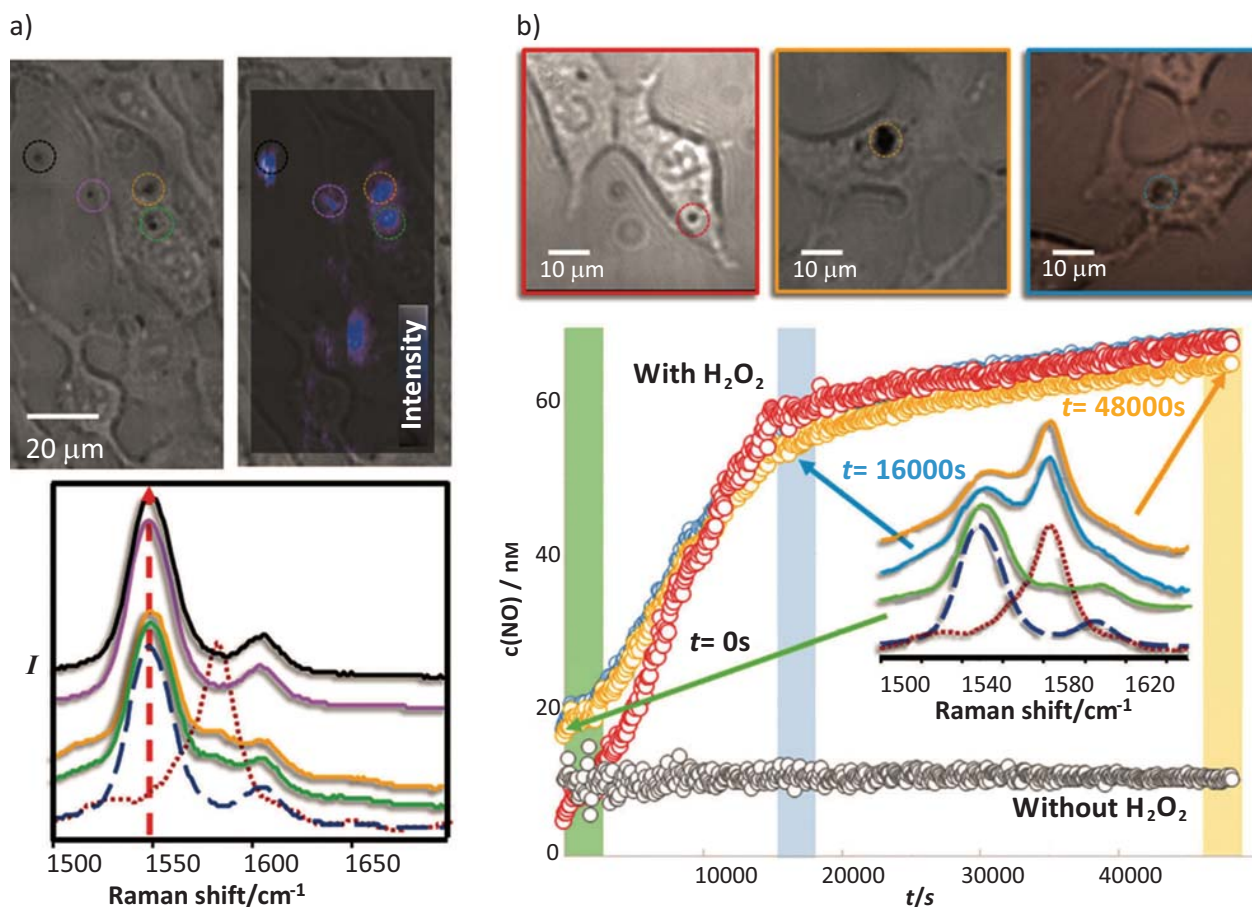


Fig. (6). Real time SERS quantification of lysosomal nitric oxide (NO) inside living cells. **a)** Transmission image and SERS mapping at 1548 cm^{-1} of the cells exposed to the NO sensors under normal conditions. The dotted circles are extra- and intra-cellular regions selected for further SERS analysis of NO. SERS spectra (bottom image) of the NO capturing molecule (ABT, dashed blue spectrum/HBT, dotted red spectrum) and of the different cellular regions. Small concentration of NO (5 and 10 nM) was detected (orange and green regions) corresponding to an increase of the HBT peak in contrast to the other two regions (black and purple) showing the bare ABT spectra. **b)** Individual capsules (red, yellow and blue dotted circles) were monitored over time to quantify lysosomal NO formation upon its induction with hydrogen peroxide (H_2O_2). The SERS detection is based on ratiometric measurements between the ABT and the HBT band [$I_{1583}/(I_{1583}+I_{1548})$]. A control sample without the addition of H_2O_2 and the SERS spectra of ABT (dashed blue line) and HBT (dotted red line) are presented for comparison. (Reprinted with permission from ref. [41]).

cles (PC) are presented as a novel SERS nanosensor for *in situ* detection of CO based on the reaction of palladacycle carbonylation (Fig. 7). Normal human liver cells and HeLa cells were used to perform the *in vivo* studies and further indicate that with the proposed SERS strategy, the detection of CO can reach concentrations as low as $0.5\ \mu\text{M}$, demonstrating its great promise for the analytical investigations of physiopathological events involved with CO.

Apoptosis, or programmed cell death detection is another highly important example of SERS-based *in vitro* sensing of cellular processes. For this, a silicon-based Ag nanoparticle (AgNPs)-decorated silicon wafer (AgNPs@Si) acts as the active and reproducible SERS *in vitro* platform for the sensitive detection of apoptotic cells at the single-cell level [66]. In the same

direction and toward the development of living cells biosensors, Au-coated nanoporous alumina-based SERS platform with increased efficient large-area, is able to detect cells presence and health status or other possible environmental condition, as demonstrated with Raman measurements in N2a living cells [20c].

Another approach that explicitly indicates the versatile character of the SERS-based strategies in cell analysis consists in stabilizer-free Ag nanoparticle-coated micropipettes as robust SERS-active microprobes for single cell detection [67]. In general, micropipettes made of pulling glass capillary can be used in microinjection of a single cell, thus, after coating with NPs, they can directly interrogate the cellular system without the use of external Raman reporters. In this way, the information from the cell itself and its chemi-

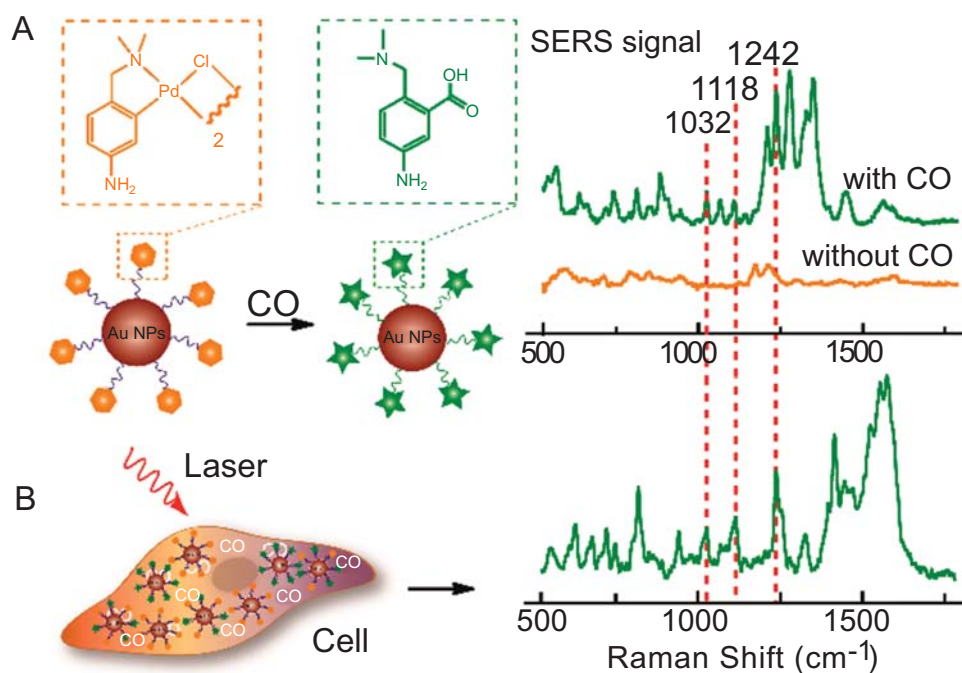


Fig. (7). SERS detection of intracellular carbon monoxide (CO): (A) Scheme of the sensor consisting of gold nanoparticles functionalized with palladacycle for CO detection. (B) Selective detection of CO inside living cells. (Reprinted with permission from ref. [65]).

cal response to the environment is obtained with great precision through SERS detection.

In vivo multiplex SERS-based biosensing is a highly promising alternative among modern diagnostic schemes. The NIR region attracts a lot of interest since is the most suitable to perform *in vivo* studies. Novel NIR SERS nanotags with multiplex capability for cancer sensing in living mouse are based on NIR Raman reporter molecules for deep tissue excitation [68]. Antibody conjugated SERS nanotags were designed to simultaneous multiplex 3 intrinsic cancer biomarkers (EGFR, CD44 and TGF β RII) in Balb/c nude female mice [69].

CONCLUSION

SERS has a huge potential of opening the landscape of current diagnostic strategies. By adequate design of nanomaterials, SERS can be a very sensitive tool for ultratrace analysis of bioactive molecules so far undetectable. The high specificity of SERS further contributes to detect very low concentration of bioanalytes. Thus, going beyond the state of the art in the early diagnosis of diseases like cancer, Alzheimer's or Parkinson's disease. Multiplexed point-of-care identification is also possible with appropriate materials. SERS is a non-invasive, *in situ* technique that do not necessarily required a big complex infrastructure and portable devices are available. To take the most advantage of this

technique in biomedicine, it is crucial to rationally design the material used for SERS. Not only because of toxicity issues but because the performance of SERS materials is significantly affected.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

P.R.G. (RYC-2012-10059, CTQ2013-45433-P[FEDER] and MDM-2014-0370), D. T. (FJCI-2015-26982) and M.S.S. (FJCI-2014-22398) acknowledge the Ministry of Economy, Industry, and Competitiveness (MINECO) and AGAUR (2017 SGR 1054) for financial support.

Funding support was also received SAF2015-73052-EXP/AEI.

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