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Recent advances of surface enhanced Raman spectroscopy (SERS) in optical biosensing

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ABSTRACT

Surface-enhanced Raman spectroscopy (SERS) is a powerful biosensing technique that combines molecular fingerprint specificity with high sensitivity, detecting trace amounts using plasmonic-based metallic nanostructured sensor platforms. SERS strategies include direct and indirect, as well as targeted and untargeted methods, depending on sample complexity and target analyte affinity. The development of SERS platforms, such as microfluidic environments, lab-on-a-fiber approaches, and paper-based immunoassays, aims at creating portable systems for point-of-care use in clinical and non-lab settings. Combining SERS with other techniques enhances measurement conditions, miniaturization, and sensitivity. This review summarizes key analytical applications of SERS in biosensing, including medicine, clinical diagnostics, environmental monitoring, food quality assessment, and biological studies.

1. Introduction

Surface enhanced Raman spectroscopy (SERS) became an attractive tool in biosensing schemes as it allows detecting the Raman fingerprint information with a high sensitivity due to the application of plasmonic metal nanostructures as sensor platforms [1–3]. Celebrating the 50th anniversary of the SERS effects, we summarize the current state of the art of this technique in optical biosensing. The inherent weak Raman signal is enhanced by 6–8 orders of magnitude as a consequence of the strong electromagnetic field enhancement on the surface of plasmonic-active nanostructures due to the interaction with light [4]. Additionally to this electromagnetic enhancement mechanism, an enhancement due to charge-transfer is achieved; however, this effect contributes only with a minor amount, i.e. 1 or 2 orders of magnitude, to the overall SERS enhancement [5]. In SERS, the orientation of the molecule towards the metallic surface is crucial as only Raman modes perpendicular to the surface are preferentially enhanced [6,7]. Consequently, the SERS signal varies for those molecules which are not adsorbed in a reproducible manner by the same moieties or binding sites onto the metal surface. As SERS-active substrates, mainly silver, gold and copper nanostructures are described in the literature [8,9]. Their fabrication is performed following chemical bottom-up synthesis, self-organization protocols as well as top-down techniques such as lithography. Their arrangements vary from isolated nanostructures in any shape, such as sphere, triangles or stars, to dimers with nanosized gaps and finally periodically and densely arranged metasurfaces. In order to meet the requirement for modern analytics, by pointing towards point-of-care applications in real biological objects, i.e. objects with complex composition, SERS sensing platforms are equipped with a functional component, e.g., with metal-organic framework structures, to attract target molecules or to repel matrix components [10,11]. Moreover, these platforms are often designed to be flexible in order to fit into

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sensor devices or directly adhere to uneven surfaces [12,13].

Therefore, within this review article, we aim to provide an overview about SERS-based biosensor platforms reported since 2020 for the interested reader, with many of them showing potential towards point-of-care testing. Moreover, relevant application fields are addressed, summarizing research work in oncology, infectious diseases and further clinical-relevant studies, as well as environmental monitoring, food quality estimation and biology-driven research tasks. Thus, a beginner within the field will be able to assess the most promising SERS detection scheme and platform for their distinct research task after studying this review article.

2. Main detection schemes in SERS

2.1. Direct and indirect SERS in untargeted and targeted sensing schemes

A SERS analysis can be carried out by using various detection schemes. Historically, the first applied sensing strategy is **untargeted direct SERS (label-free SERS)**, where all present molecules are allowed to interact with the metallic surface and those molecules with the highest affinity towards the metallic surface will dominate the SERS response. In case of multiplexed analysis, Raman modes can be assigned to each target by direct comparison with SERS spectra of individual targets acquired in identical experimental conditions [14]. **Untargeted SERS analyses** of biological samples, in which as many sample constituents as possible should be detected, always rely on a direct detection. Even in complex mixtures as biofluids, the observed SERS spectra will show the bands of all those species with a strong affinity for the metal substrate. The SERS intensity, in fact, will depend much more on affinity for the metal than on concentration.

Most recent SERS applications in SERS sensing are **targeted** at the detection or quantification of one or more specific analytes. Such targeted analyses most often use an **indirect detection strategy (label-based or labeled SERS)**, in which the SERS signal observed is not from the targets themselves, but from SERS tags (constituted by molecular "reporters" or "labels" on a plasmonic nanostructure) that get selectively conjugated to the target via a recognition element [15]. The principle construction of a SERS tag is provided in Fig. 1. Molecular dyes and thiolated aromatic molecules are mainly used as Raman reporters due to their large Raman cross-section, good interaction with the surface of metal nanoparticles (NPs), and stability with respect to photobleaching. Protective layers (e.g., a thin layer of SiO_2) are sometimes used to prevent (i) desorption of the Raman reporter from the NP surface, and (ii) an uncontrolled aggregation of the SERS tags. Finally, a recognition element layer of immunoreagents or aptamers provides selectivity for the targets. The most common example of indirect targeted SERS strategy implementation is lateral flow immunoassays (LFA), in which antibodies are used to selectively capture the target analytes and conjugate them with SERS tags, instead of fluorescent or colored tags [16]. Other examples of recognition elements are nucleic acid sequences or molecularly-imprinted polymers (MIPs). In case of multiplexed analysis, target identification is straightforward once the associations between labels and recognition elements are known. Another indirect SERS sensing strategy aims at detecting spectral variations due to structural or conformational changes of the recognition element itself, occurring upon target binding [17–19]. Targeted analyses, however, can be also carried out by using a **direct detection strategy (label-free SERS)**, in which the SERS bands observed are those due to the analytes themselves. This kind of detection strategy is used for those targets having a high affinity for the metal used as SERS substrate, so that spectral interferences due to the co-adsorption of other species present in the sample is minimized [19]. Within the following, targeted SERS sensing strategies employing various recognition elements (antibodies, aptamers and MIPs) are further introduced in more detail.

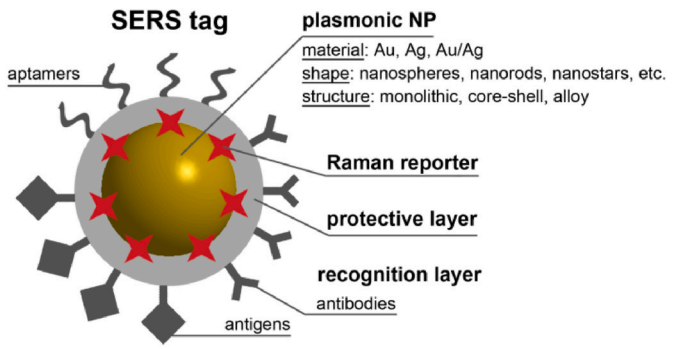

Fig. 1. Illustration of the architecture of a SERS tag.

2.2. Indirect targeted SERS with immunoreagents as specific recognition elements

Typically, SERS-based immunoassay requires a solid substrate modified with **immunoreagents** (antibodies or antigens) and the SERS immunoprobe (SERS tags), which provide SERS signal to determine the analyte. The sensitivity of SERS immunoassays is mainly determined by enhancing properties of the metal NPs; thus, careful design of the NP shape and structure can improve the sensitivity. SERS tags have several advantages compared to the labels of other types (e.g., fluorescent and colorimetric). First, SERS tags can be easily used for extending the advantage of a multiplex analysis due to very narrow SERS peaks (15–25 cm^{-1}), allowing the detection of a large number (i.e. 10-15) of different tags in one experiment, by using a single laser for signal generation. Second, the SERS signal is less susceptible to photobleaching and quenching. Finally, SERS-based immunoanalysis is up to a 1000-fold more sensitive compared to the colorimetric immunoanalysis due to the much higher optical efficiency of the SERS tags [20]. Additionally, SERS tags can also be used as immunolabel for mapping of cells, tissues, etc. [21,22] Depending on the analyte properties, the analysis can be carried out in different formats, as illustrated in Fig. 2. Non-competitive

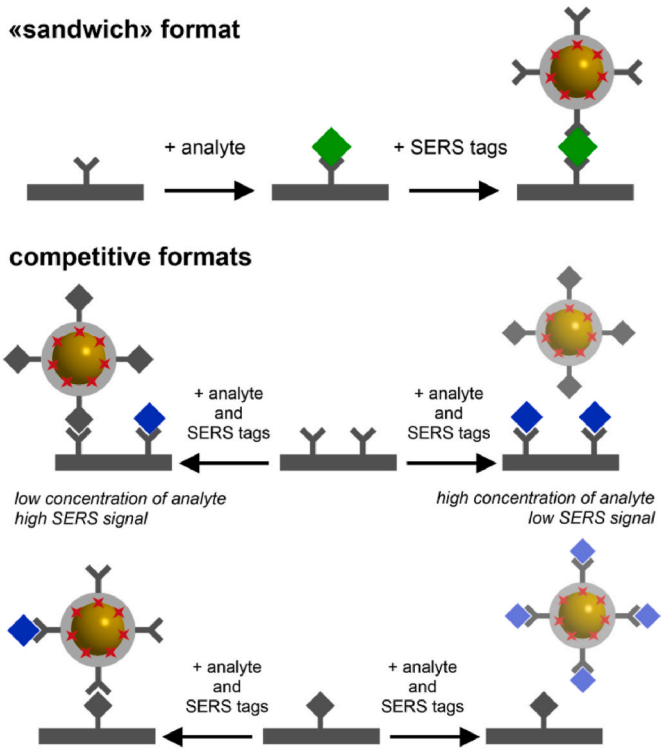

Fig. 2. Schematic illustration of main formats of SERS immunoassays.

("sandwich") format is used to detect relatively large molecules (e.g., proteins). In this case, the analyte is sandwiched between two clones of monoclonal antibodies specific to different fragments of the analyte [23,24]. Competitive format is suitable for capturing small analytes with only one binding site. In this case, the analyte molecules compete with SERS tags for binding to the antibodies [25]. Disadvantages of SERS-based immunoassays are the same as for other antibody-based assays: complexity and high cost of obtaining antibodies, the need to develop antibodies for each individual analyte, inability to obtain antibodies for some small molecules due to the lack of immunoreactions.

2.3. Indirect targeted SERS employing aptamers for specific recognition

Aptamers consist of single-stranded nucleotide sequences that can bind to specific target by folding into specific three-dimensional structures. Aptamers are more stable and nearly 10-times smaller than antibodies that imply less time to reach equilibrium with target analyte and a more organized arrangement on the SERS substrate. Compared to antibodies, aptamers can be produced for various targets (from small molecules [26,27] to cells) [28] in large quantities and with high reproducibility. The aptamers can also be modified with functional groups which increase interaction with SERS substrates (e.g., SH-groups) [26,29,30]. Aptamer-based SERS assays can be realized in both direct and label-based formats (see Fig. 3). In the label-free format the aptamer molecules directly immobilized on a SERS-active surface selectively concentrate analyte molecules near the surface (Fig. 3a). For example, Cho et al. [26] demonstrated that the modification of SERS substrate with the aptamer resulted in ~20 % increase in the SERS signal of bisphenol A compared to the unmodified SERS substrate. However, because nucleobases inside aptamers also generate SERS signal, the analyte can be directly detected only if the intensity of its Raman signal is stronger than that of the aptamer. In contrast to the SERS immunoassays, this format is possible due to the smaller size of aptamers compared to antibodies, i.e. the distance between the analyte and the SERS substrate is small enough to enhance Raman signal of the analyte. Cho et al. [26] also successfully tested SERS substrate coated with several aptamers for different analytes that allows for simultaneous determination of several analytes with no competition for binding to the SERS-active sites.

Label-based formats of aptamer-assisted SERS assays can be realized in different ways. For example, the aptamer molecules attached to the SERS-active surface can be also used as the Raman reporter (Fig. 3b). In this case, an addition of the target analyte causes changes in the conformation and, consequently, SERS spectrum of the aptamer. Thus, the analyte concentration can be found by monitoring these changes [30]. However, different aptamers can have approximately the same SERS spectra if they contain the same nucleobases and the amounts and ratios of the nucleobases are similar (while having different sequences), and this fact restricts applicability of aptamers as labels for multiplex analysis. To overcome this limitation, the aptamer molecules chemically modified at one end with a Raman reporter (mainly dyes) have been also proposed for SERS analysis, as shown in Fig. 3c. For example, Pramanik et al. [29] developed SERS substrate modified with Rhodamine 6G (R6G)-labeled aptamers and its analytical signal decreases upon binding of the analyte molecules to the aptamer ("on/off" format of the signal changing) due to an increase in the distance between the Raman reporter (R6G) and the SERS substrate surface. Moreover, the use of hybridization of two complementary aptamer fragments has been also proposed to prepare label-based assays with "on/off" format. For example, Chen et al. [31] proposed an assay based on the metal NPs coated with two aptamer molecules which are hybridized into a complex (Fig. 3c). One aptamer is chemically bonded to the NP surface and the second one is modified with the Raman reporter so that the SERS substrate modified with the hybridized fragments has an intense SERS signal. Addition of the analyte molecule leads to detachment of the second aptamer with higher affinity to the analyte and, consequently, reduction of the Raman reporter signal.

Some of proposed label-based assays are quite similar to SERS-based immunoassays and they are realized in "sandwich" [28] and competitive [27,32] formats using SERS tags to generate the analytical signal (Fig. 3d and e). Moreover, antibodies and aptamers can be used together, e.g., in "sandwich" format of SERS analysis. For example, Li et al. [33] used antibody-modified Fe_3O_4 as a capture probe and aptamer-modified SERS tags for label-based detection of bacteria cells. Lastly, Lu et al. [32] proposed another version of competitive label-based assay that is based on agglomerated SERS tags with a strong SERS signal in absence of the analyte (Fig. 3e). The agglomeration of the aptamer-modified SERS tags occurs after hybridization with a specific

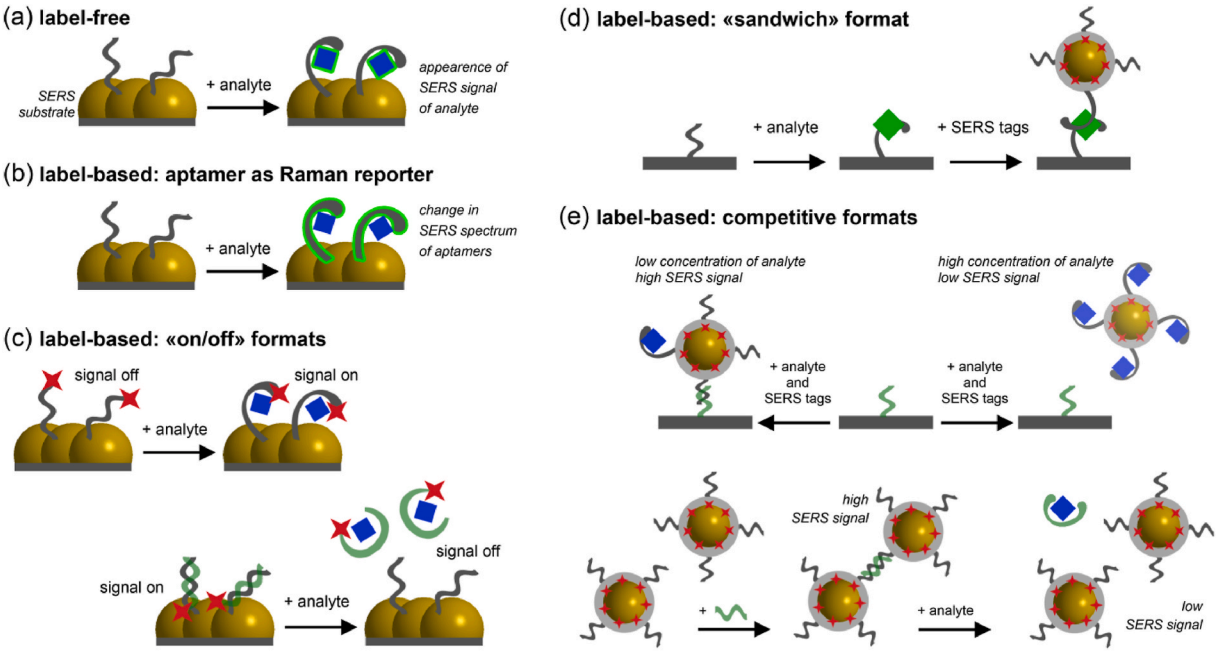

Fig. 3. Schematic illustration of main formats of aptamer-based SERS assays.

bridging aptamer that leads to the formation of abundant hot-spots with intense SERS signal. The bridging aptamer is also capable for selective interaction with the analyte, and addition of the analyte molecules leads to the dissociation of the SERS tag agglomerates. As a result, the number of hot-spots is decreased, and the signal intensity produced by SERS tags is also decreased.

Thus, we should state a strong progress in the SERS analysis assisted with recognition compounds. For example, all label-based formats can be used for multiplex analysis thanks to availability of various Raman reporters (by themselves or as part of SERS tags), which have their own set of characteristic peaks. Thus, the number of analytes, for which label-based assays have been proposed, is limited only by the ability to obtain the recognition elements (both antibodies and aptamers). The use of magnetic nano- or microparticles as supports for immunoreagents or aptamers additionally facilitates analyte separation from the sample matrix by an external magnetic field [23,33]. Moreover, magnetic-based separation enables to concentrate and agglomerate SERS tags further improving sensitivity of the analysis. Finally, some formats of immunoreagent- and aptamer-assisted SERS analysis can be realized in the form of LFA on nitrocellulose membrane with zone of SERS tags and control and test zones with immunoreagents or aptamers [20,25].

2.4. MIP-assisted SERS assays

Molecularly imprinted polymers (MIPs) are synthetic recognition elements which were also used to improve selectivity of the SERS analysis. Generally, MIP-SERS substrates consist of a SERS substrate coated with a thin MIP shell from which template molecules were completely removed from recognition sites during the substrate fabrication, leaving sites with a high affinity for the analyte available for binding [34–36]. MIP-SERS substrates are usually employed for direct SERS analysis; however, if the analyte does not have its own SERS signal, a competitive label-based SERS format can also be realized. For example, Arabi et al. [36] used addition of a Raman reporter (diethylthiatricarbocyanine iodide) after adsorption of the target analyte (trypsin) on the surface of MIP-SERS substrate. In this assay, an increase in trypsin concentration leads to a decrease in the intensity of analytical signal due to reduction of the number of adsorption sites in the MIP shell, which are suitable for adsorption of the Raman reporter molecules. Compared to antibodies and aptamers, MIP-SERS substrates can be reused several times after washing step. For example, Kou et al. [34] demonstrated that MIP-SERS substrate could be used for analysis 7 times without decrease in the intensity of analytical signal. Additionally, MIPs as recognition elements have high batch-to-batch reproducibility and can be used under extreme conditions (e.g., extreme pH, organic solvents). Another important advantage is the ability to facilitate the adsorption of analytes with poor affinity to standard SERS substrates (e.g., hydrophobic analytes). However, the recognition and binding capability of MIPs is not as good as that of antibodies or aptamers. Consequently, the selectivity of MIPs can be reduced due to nonspecific adsorption of low molecular weight impurities. Importantly, impurities may not produce their own SERS signal, but they may occupy and block the recognition sites of MIP-SERS substrate leading to a decrease in the SERS signal of the target analyte [35]. The stability of the properties of MIP-SERS substrates is also questionable. For example, one study demonstrated that the signal intensity produced by the MIP-SERS substrate is stable during 10 months of storage [34], while another study showed 30 % decrease in the intensity after 30 days of storage [35].

2.5. Bioorthogonal SERS

An alternative label-technique, recently applied in SERS, is **bioorthogonal** labeling, where chemicals are used in biological conditions without inducing alterations but only labeling/modifying the molecule of interest, has attracted great attention. The great potential lies in being a flexible and robust tool for visualization detection and diseases diagnosis through cell imaging, biomarkers-analysis in body fluids and tissues studies. SERS reporter-labeled nanoparticles, derived from combining nanoparticles with nanoprobe yielding narrow vibrational modes in biological Raman-silent region (1800-2800 cm^{-1}), result in an important tool for the development of optical bio-assays due to their high SERS sensitivity and properties of multiplexing. Especially azide, alkyne and nitrile moieties have been anchored with success to both silver and gold nanoparticles [37]. In their review, Qiu et al. report the latest progress in the applications of bioorthogonal SERS for living-cells imaging and cancer-tissue detection, highlighting the possible identification of proteins, nucleic acids and other intracellular components [38]. Furthermore, in addition to mentioning the drawbacks due to the use of nanotags in biomedicine, they discuss the strategies in using bioorthogonal SERS as imaging-guided means for phototherapy and surgery, forecasting a positive contribution for biomedical purposes. Regarding living-cell studies, a recent work illustrates a novel bioorthogonal SERS nanotag, composed by gold nanoflowers and 4-mercaptobenzonitrile for cancer-cell imaging application [39]. Here, with high SERS sensitivity and accuracy, HeLa cells imaging recognition from co-cultured cancer cells has been demonstrated, opening a new route for cancer theranostics. The spectral analysis of liquid biopsy has been shown to have great potential as diagnostic tool, especially in biomarkers detention and disease screening. Dallari et al. proposed a bioorthogonal SERS method, based on embedded-nanoprobe in gold nanoparticles bilayer, for Amyloid β-peptide 1−42 quantification in Alzheimer's-patient cerebrospinal fluid, demonstrating the possible potential of this approach in biomedical research [40].

3. SERS-based biosensor platforms

In order to design powerful SERS sensing platforms, SERS is combined with various techniques, which will be introduced within this chapter. These techniques are differently suited for point-of-care applications; however, the individual analytical task might estimate the best suited SERS sensing platform when the combination with other techniques is required.

3.1. Microfluidic SERS

By applying microfluidic setups in combination with SERS, continuous flows are generated providing a perfect environment to ensure reproducible measurement conditions [41]. In continuous flow arrangements, solid SERS substrates are integrated, e.g. as chip platform or directly bound to the channel walls; however, due to the enrichment of the analyte over time at the nanostructured sensor surface, the so-called memory effect is observed. Moreover, the mixing efficiency in continuous flow architectures might be too low when a previous mixing of several sample solutions is required prior the SERS analysis.

To overcome the limitations of continuous flow cell in SERS measurements, flow cells allowing for a segmented flow or droplet-based systems are available, employing mostly colloidal nanostructures as SERS substrate [41]. Here, a lower memory effect is observed as colloids are freshly mixed with the analyte of interest and after the SERS measurement, the SERS-active nanostructures together with the analyte molecules are transported out of the chip platform. Through specially designed channel architectures, e.g. meandric channels, a most efficient mixing is achieved within the droplets or segments. The metallic colloids applied in these segmented flow arrangements could be prepared freshly *in-situ* or pre-prepared outside the chip platform.

In case of multiplexing capabilities of microfluidic SERS schemes, 3 platforms could be described: (i) spatial multiplexing whereas recognitions elements are spatially distributed across the chip platform and a positive binding event between analyte and SERS tag can be observed depended on its positions, e.g. in the microfluidic channels; (ii) barcode-enabled multiplexing by employing SERS tags equipped with different Raman reporter molecules and antibodies or aptamers being specific for

individual analytes; (iii) label-free or direct multiplexing where all molecules with high affinity towards the metallic surface can contribute to the SERS signal, supported by chemometrics and/or artificial intelligence (AI) algorithms for readout [42]. In Fig. 4, a summary of the most applied multiplexing detection schemes in microfluidic SERS is provided.

Finally, microfluidic SERS platforms are described for isolation and sorting, for example analyzing small extracellular vesicles, employing the label-free or direct SERS approach as well as SERS nanotags [43]. The range of applications in microfluidic SERS is broad, covering topic such as food contaminant detection [41], bioassays [44] and oncology [42,43].

3.2. Lab-on-a-fiber SERS

Cutting-edge fiber optic technology enables the interaction of light with functionalized fiber surfaces at remote locations to develop a novel, miniaturized and cost-effective lab on fiber technology for bio-sensing applications. Recent developments in the field of nanotechnology provide innumerable functionalization methods to develop selective bio-recognition elements for label free biosensors. These methods may be easily integrated with fiber surfaces to provide highly selective light-matter interaction depending on various transduction mechanisms. The design and performance of these biosensors is measured in terms of operating range, selectivity, response time and LOD [45].

Gu et al. [46] proposed a seed mediated growth of AuNPs monolayer on a fiber facet for ultrasensitive in-situ SERS detection of methylene blue, crystal violet and R6G up to 10^{-9} M with the experimental set up shown in Fig. 5. These optrodes geometry can render a reliable and promising *in situ* SERS detection platform that can efficiently cater to the ever-increasing need for sensitive and remote monitoring of various biological and chemical analytes. However, repeatability, excitation/collection efficiency and fluorescence noise are some of the concerns to be taken care of in this type of sensing platforms.

Managò et al. [47] studied the influence of the geometrical configuration of SERS-active self-assembling gold nanostructures on the interaction with biological targets of different sizes, aiming at large-scope SERS lab-on-fiber optrodes composed of highly efficient SERS substrates integrated onto optical fibre tips. By using nanosphere lithography, they fabricated three types of highly ordered and reproducible SERS-active substrates. To correlate the SERS response to the steric hindrance of the biological target, they experimentally analysed and compared the SERS spectra of three representative biological probes, i.e., ultralow-molecular-weight molecules of biphenyl-4-thiol (small molecule, 186.27 Da), bovine serum albumin (BSA, medium molecule, 66.5 kDa) and red blood cells (RBCs, diameter <10 μm, complex target). All SERS-active substrates provided the Raman fingerprint of the biological targets, but the SERS intensity was dependent on the substrate geometry and its specific steric interfacing with biological targets. A full three-dimensional numerical analysis was carried out by means of the finite element method to gain insight into the electric field distribution and hot spot distribution of the fabricated structures. By correlating the electric field distribution with the SERS response, they concluded that the most efficient target-based architectures should feature not only intense but also accessible hot spots. They also optimized a Raman readout system for SERS optrode operation with efficient illumination and collection via an optical fiber probe in direct and remote configurations as shown in Fig. 5.

3.3. Portable SERS for point-of-care testing

In the last years, SERS is gaining popularity as a method for diagnosis at the point-of-care. ELISA, PCR, and RT-PCR have been used to detect respiratory viruses for pandemic control, but have limitations when it comes to point-of-care testing. Recently, the design of Raman spectroscopic systems has been simplified from a complicated design to a small and easily accessible form that enables point-of-care testing. By instrumentation and commercialization advancements, the advent of the portable handheld SERS devices creates a fast, accurate, practical, and cost-effective analytical method for virus detection, and would continue to attract more attention in point-of-care testing [48].

For example, Leong et al. [49] developed a handheld breathalyzer to identify COVID-19 patients within 5 min attaining >95 percent specificity and sensitivity across 501 subjects from a clinic case-control research study in Singapore as shown in Fig. 6. The SERS chip inside this breathalyzer consists of an aluminum plate with three zones with deposited silver nanocubes modified with different molecular receptors for interaction with different breath metabolites. Using the SERS-based breathalyzer, the authors recorded changes in vibrational signatures after interaction of molecular receptors and breath metabolites, and developed a classification algorithm for high-throughput spectrum studies based on the partial least squares discriminant analysis, which was integrated with portable Raman instruments to provide an instant result without sample preparation.

Inflammatory biomarkers are closely related to infectious diseases. However, traditional clinical tests are unable to achieve rapid and accurate detection of these biomarkers on-site due to complex experimental operation, expensive equipment, and long test duration. Li et al. [50] proposed a LFA strip based on SERS nanotags for the simultaneous and quantitative detection of dual infection biomarkers, serum amyloid A (SAA) and C-reactive protein (CRP), respectively. The detection principle is illustrated in Fig. 7. In practice, mesoporous silica ($mSiO_2$)-coated AuNPs were used as the SERS substrate. Mercaptobenzoic acid (MBA) was embedded in the internal gap between AuNPs

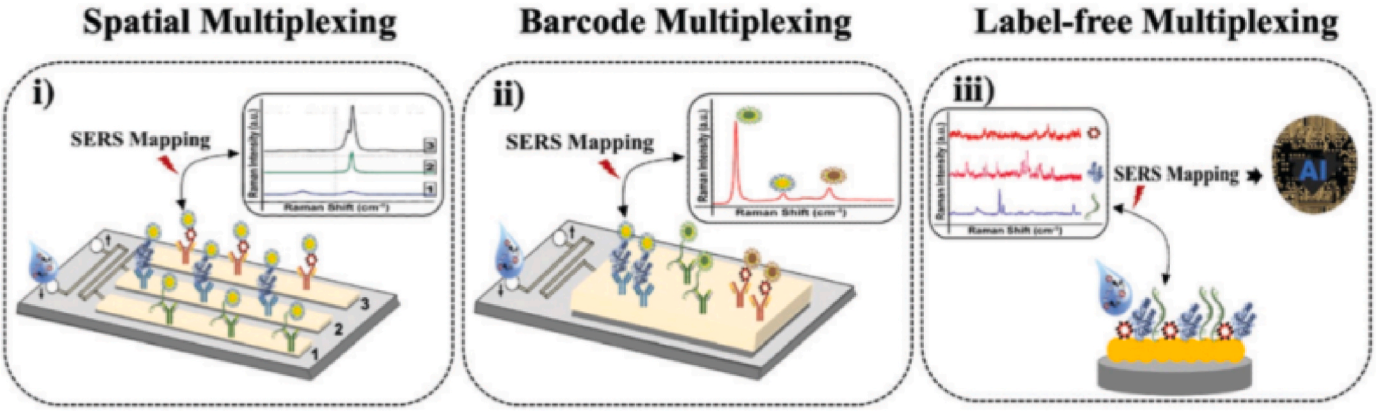

Fig. 4. Multiplexing SERS platforms by employing microfluidic platforms [42]. Reproduced under a Creative Commons Attribution-NonCommercial 3.0 Unported Licence.

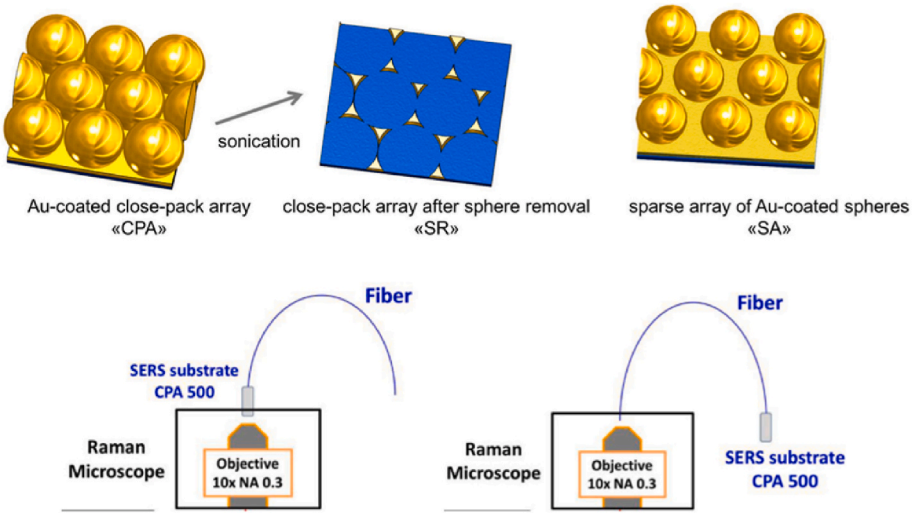

Fig. 5. (top) Three types of SERS-active substrate under investigation. (bottom) Schematic of the SERS test with a fibre optic probe in direct (left) and remote (right) configurations. Reprinted from [47], Copyright 2021, with permission from Elsevier.

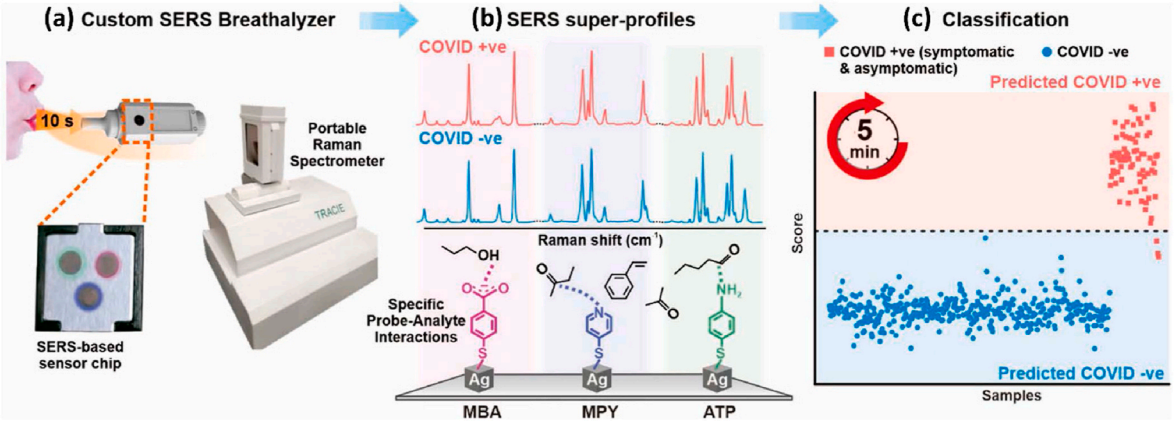

Fig. 6. A flow-diagram of the SERS-based method for detecting COVID-positive patients utilizing volatile organic molecules in their breath (BVOCs) [49].

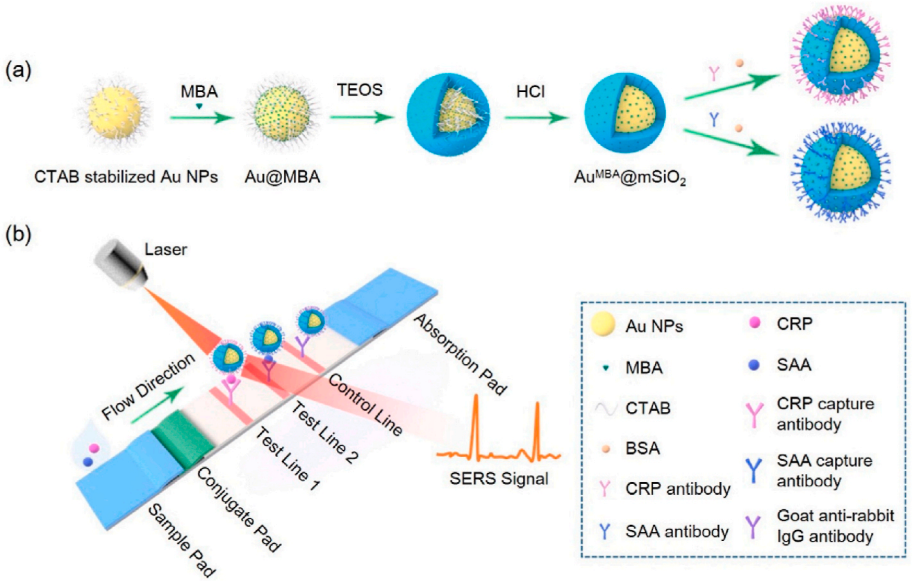

Fig. 7. (**a**) The preparation flow chart of the core-shell AuMBA@mSiO$_2$ nanotags; (**b**) the principle of the SERS-LFA strip for detection of SAA and CRP [50]. Reproduced under Creative Commons Attribution License 4.0 (CC BY).

and the $mSiO_2$ shell to prepare AuMBA@$mSiO_2$ NPs, onto which SAA and CRP antibodies were modified to prepare two AuMBA@$mSiO_2$ SERS nanotags. The Raman intensities of the test and control lines were simultaneously identified for the qualitative detection of SAA and CRP, with limits of detection (LODs) as low as 0.1 and 0.05 ng/mL for SAA and CRP, respectively. Finally, aiming at point-of-care testing applications, they used a smartphone-based portable Raman spectrometer to quantitatively analyze the SERS-LFA strips. The Raman signal could still be accurately detected when the concentration of SAA and CRP was 10 ng/mL, which is lower than the LOD required in clinical practice for most diseases. Therefore, taking into account its simple operation and short analysis time, by using a portable Raman spectrometer which can be equipped with a 5G cloud-based healthcare management system, the current strategy based on SERS-LFA provides the potential for the quick and on-site diagnosis of infectious diseases such as sepsis, which is of great significance for medical guidance on the treatment of widely spread infections in remote areas that lack well-developed medical resources [50].

3.4. Electrochemical SERS

Electrochemical surface-enhanced Raman spectroscopy (EC-SERS) is a method that combines electrochemistry and SERS to achieve enhanced sensitivity and specificity. In the electrochemical cell, molecules close to the electrode undergo either oxidation or reduction, which is detected by the current passing through the electrodes. However, this process does not provide information about the specific molecules involved. On the other hand, SERS is sensitive to molecules in close proximity to the surface, and its signal is improved when the target molecule adheres in a higher quantity to the surface [51]. Applying voltage to the SERS substrates, which act as the working electrodes, leads to observable changes in the SERS spectra. This occurs by bringing the molecules closer to the electrode surface through an electrochemical double layer or altering the absorbed molecular orientation on the surface.

In EC-SERS, electromagnetic and static electrochemical fields coexist within the electrochemical cell environment, influencing chemical and physical enhancements. This method has been applied to investigate the adsorption and reaction of various species in aqueous solutions, including inorganic molecules, organic compounds, and biomolecules [52].

By carefully controlling the electrode potential, EC-SERS can be adjusted to maximize surface enhancement, creating a suitable surface for biomolecule interactions. This is particularly useful when dealing with charged species. EC-SERS has been gratefully utilized to detect and quantify in various applications ranging from small organic molecules, drugs in pharmaceuticals, and complex biomarkers to proteins and bacterial cells. It is also utilized in complex matrices such as body fluids directly, and in environmental and food safety applications [53].

Overall, EC-SERS shows promise for applications in biological and biomedical research, offering a versatile approach to diagnostic techniques. Although EC-SERS provides more spectral richness and fewer signal fluctuations than normal SERS, it must be optimized and standardized to become a reliable tool.

3.5. Surface enhanced spatially offset Raman spectroscopy (SESORS) and surface enhanced coherent anti-Stokes Raman spectroscopy (SECARS)

The breakthrough in deep probing of tissue with Raman spectroscopy emerged with the advent of spatially offset Raman spectroscopy (SORS). The approach relies on the fact that deeper penetrating photons statistically tend to migrate laterally from the illumination zone on the sample surface, whereas those photons that have scattered back to the surface from shallower depths have had less opportunity to travel laterally. The typical presence of surface-to-air interfaces accentuates this effect by preferentially facilitating loss of the photons propagating through near-surface layers, as any laser photon reaching the interface is immediately and irretrievably lost. Collecting Raman photons at the surface of the sample away from the laser illumination zone therefore biases the detected signal towards deeper zones within the sample. The separation between the illumination and collection zones is termed spatial offset. Using varying spatial offsets can help elucidate the chemical composition of individual layers in a sample. SORS has general applications in biological areas, such as identifying biomarkers or quantifying biological subcomponents. A more advanced variant, ring illumination SORS, uses a ring-shaped laser illumination zone and Raman signal collection from the center. This allows for increased illumination laser power for larger spatial offsets, benefiting *in vivo* applications where intensity is typically restricted by laser safety thresholds [54].

In order to increase the sensitivity in deep tissue investigations, SORS is combined with SERS by employing SERS-active nanoparticles and tags, creating surface enhanced spatially offset Raman spectroscopy (SESORS). The ability to precisely determine the specific depth at which the SERS tags are located without any prior knowledge remains a significant challenge and the means to do so is especially important for *in vivo* applications where those tags will be distributed at different and unknown depths, e.g. within a cancerous lesion [55]. Using SORS and transmission Raman, Mosca et al., reported a viable and robust method capable of predicting the depth of both a single buried object and SERRS tags through turbid phantoms by monitoring the relative intensity of two Raman bands exhibiting differential absorption by the matrix [56]. The results demonstrate a potential effective means of calculating the depth at which SERRS tags are localized *in vivo* as well as determining the optimum distribution of laser radiation around a sample in photothermal therapy. However, longer propagation distances result in larger relative Raman band intensity distortion of the target signal due to tissue differential absorption. This is a result of water and/or lipids absorbing the two monitored Raman bands differently [56]. It has also been demonstrated that it is possible to precisely predict the depth at which SERS tags were buried in a turbid phantom (0.5 % intralipid) and simultaneously monitor changes in pH of the media surrounding them [57].

Moody et al. [58] reported the ability to resolve spectral signatures from individual neurotransmitters and mixtures of neurotransmitters at physiologically relevant concentrations using inverse (ring illumination) SORS. This was achieved using agarose gel which was then embedded within the *ex vivo* rat skull to create a brain tissue mimic. Using these phantoms, SESORS established LODs for a range of neurotransmitters at physically relevant concentrations; melatonin (100 nM); serotonin (400 nM); dopamine (1 mM); norepinephrine (400 nM); and epinephrine (800 nM). In addition, the brain of a sacrificed mouse was spiked with serotonin and gold NPs to demonstrate the potential of SESORS to detect neurotransmitters through the skull, (Fig. 8). This represents a notable step towards the use of SESORS for the *in vivo* detection and continual monitoring of varying concentrations of neurotransmitters in a living system. However, it is important to note the challenges associated with the detection of low concentration species directly using SESORS *in vivo* due to complex nature of biological fluids in which several components, e.g. proteins, will compete with the neurotransmitters in order to bind to the nanoparticles surface.

Because point-by-point SERS mapping is time-consuming and could take hours for large-scale imaging, Zong et al. [59] demonstrated a time-lapse three-dimensional wide-field surface-enhanced coherent anti-Stokes Raman scattering (WISE-CARS) microscopy for the monitoring of SERS nanotags in live cells, shown in Fig. 9, and the label-free detection of bacterial metabolites. The WISE-CARS microscope achieved an imaging speed of 120 fps for a field of view of 130 × 130 μm^2. By spectral focusing of femtosecond lasers, a hyperspectral WISE-CARS stack of 60 frames can be acquired within 0.5 s, where over one million Raman spectra are parallelly recorded with a spectral resolution of 10 cm^{-1}.

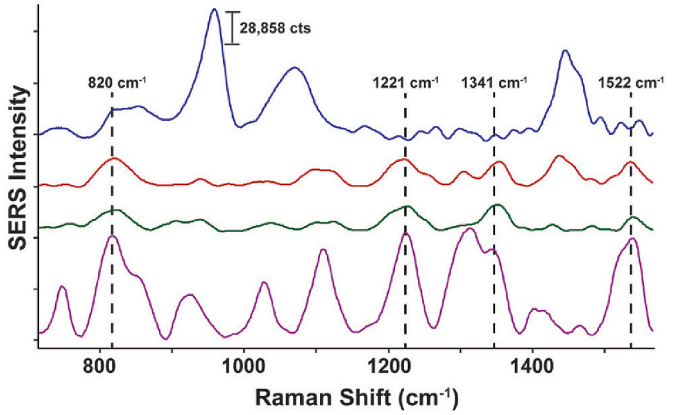

Fig. 8. *Ex vivo* detection of 100 mM serotonin through the skull of a mouse using SESORS. A SORS spectrum of the mouse brain through the intact skull without the injection of serotonin or gold NPs (top spectrum, blue). A SESORS spectrum of the mouse brain following the injection of 100 mM serotonin at a 0 mm offset (red) and a 1 mm offset (green). The SORS spectrum of the mouse brain before the injection of NPs was subtracted from the offset spectra. Data was acquired using a laser wavelength of 785 nm, 90 mW, t = 120 s. The SERS spectrum of 100 mM serotonin on AuNPs at pH 2, 785 nm, 5 mW, t = 30 s (purple) [58].

4. Application of SERS biosensing approaches

In the preceding chapters, the SERS-based detection schemes and sensor platform concepts were introduced. SERS is a versatile technique that can be applied in a multitude of ways, including direct non-targeted and indirect targeted SERS methods, as well as in combination with microfluidics, optical fibers, portable approaches for analysis at the point of care, using electrochemistry, which allows for potential-dependent investigations. Finally, SERS can be combined with spatially-offset approaches to address deeper tissue layers and non-linear Raman approaches for ultrasensitive detection schemes. All of the aforementioned approaches are suitable for addressing analytical questions that arise in biosensing. Fig. 10 provides an overview of the most commonly addressed SERS applications in biosensing. The researcher within the field of SERS will find inspiration in chapter 1 and 2 to construct a powerful SERS platform and detection scheme, which can be further applied to a relevant biosensing application task, to which examples and an introduction are provided in the following subchapters.

4.1. SERS application in medicine

4.1.1. SERS in oncology

Cancer diagnosis holds immense significance in healthcare as it is the crucial first step towards successful treatment outcomes and improved patient outcomes. The timely and accurate diagnosis of cancer enables healthcare providers to implement appropriate treatment strategies, thereby increasing the chances of successful outcomes for patients. Additionally, a proper cancer diagnosis facilitates personalized treatment plans tailored to individual patients, ensuring that they receive the most effective care and reducing the risks of unnecessary interventions. SERS has emerged as a promising tool in the field of cancer diagnosis and treatment due to its high sensitivity and ability to detect molecular changes associated with cancer progression. The importance of SERS in cancer lies in its potential to provide early and accurate detection of cancer, enabling timely intervention and improved patient outcomes. One key advantage of SERS in cancer diagnosis is its ability to detect biomarkers at ultra-low concentrations, which traditional diagnostic methods often fail to achieve. Biomarkers are specific molecules associated with cancer development, and their presence in bodily fluids can indicate the presence or progression of cancer. With the sensitivity of SERS, even minute concentrations of biomarkers can be detected, making it a valuable technique for early cancer detection. By identifying cancer at early stages, clinicians can provide immediate treatment, leading to better prognosis and increased chances of survival [60,61]. For cancer diagnostics, any protein, gene, cell-derived small vesicle or other molecule produced by cancer cells has been considered as cancer biomarker [62,63]. In Fig. 11, an overview figure is provided about important cancer marker as well as investigated body fluids and patient samples targeted by SERS-based detection schemes.

In recent years, significant attention has been directed in developing SERS-based assays for cancer genomic markers, resulting from mutations in nucleic acids. The potential of SERS for detection of miRNAs-based biomarkers in liquid biopsy has been specifically highlighted [64]. Moreover, SERS-based working principles for DNA point mutation and DNA methylation detections have been reported, together with the estimated LOD values. In the end, the authors pointed out the need to further improve the protocols towards the development of genomic SERS sensors.

Also, SERS is an emerging analytical tool for sensitive detection and characterization of cancer extracellular vesicles (EVs). These are small spherical structures released by cells that play a crucial role in intercellular communication and, thanks to their own proteins and nucleic acids, they provide valuable insights into the underlying disease pathology. Last promising progress about investigation cancer-derived EVs through means of label-aid and label-free SERS have been summarized [65]. Recently, significant papers have been published regarding EVs in specific cancer types. For instance, SERS allows the phenotyping of lung cancer-related EVs in plasma, allowing a differentiation between cancer patients and healthy controls and identification of lung cancer-related markers, with help of machine learning algorithms [66]. Again, through a longitudinal study of bronchoalveolar fluid, small EVs secreted by non-small cell lung cancer have successfully been detected using SERS [67]. A study, regarding ovarian cancer-derived EVs, illustrated successful SERS immunocomplex assembling strategies for a highly sensitive and specific detection of EVs in plasma [68]. SERS has also emerged as a valuable tool for the analysis of small class of EVs, namely exosomes. They are secreted by nearly all cell types and carry their own biocomponents and they have been implicated in numerous physiological and pathological processes, including cancer development and progression. An overview of direct and label-based SERS analysis for cancer exosomes and their subcellular components detection has been provided [69]. Combining these SERS-based analytical tools is an optimal method with complementary advantages towards achieving precise medical diagnosis.

The ability of SERS to analyze body fluids, such as blood and saliva, provides a non-invasive approach for cancer diagnosis and identify protein tumor markers, which results crucial for early detection and personalized treatment. Direct, untargeted SERS analysis of serum, in combination with machine learning, has recently allowed discrimination between colon cancer patients and a control group [70]. For untargeted SERS analyses of biofluids, a crucial (and often underestimated) aspect is the correct interpretation of bands in terms of metabolites. Often, bands are assigned on the basis of normal Raman spectra, or SERS spectra collected on different substrates, while a correct assignment can only be achieved via a direct comparison between the biofluid SERS spectrum and the SERS spectra of single metabolites acquired with the same experimental conditions. Targeted direct SERS detection in body fluids is also possible: for instance a novel approach, combining SERS and molecular dynamics simulations, has assured the detection and interpretation of well-known salivary cancer markers (i.e. IL-8 and lysozyme) for point-of-care diagnosis of head and neck cancer [71]. This combined workflow has the potential to opening up a new route in deeply investigation of biomarkers in body-fluids. Lastly, bladder extracellular fluid has been analysed through SERS method allowing the differentiation between cancerous and normal human bladder tissues [72]. In general, when working with complex samples

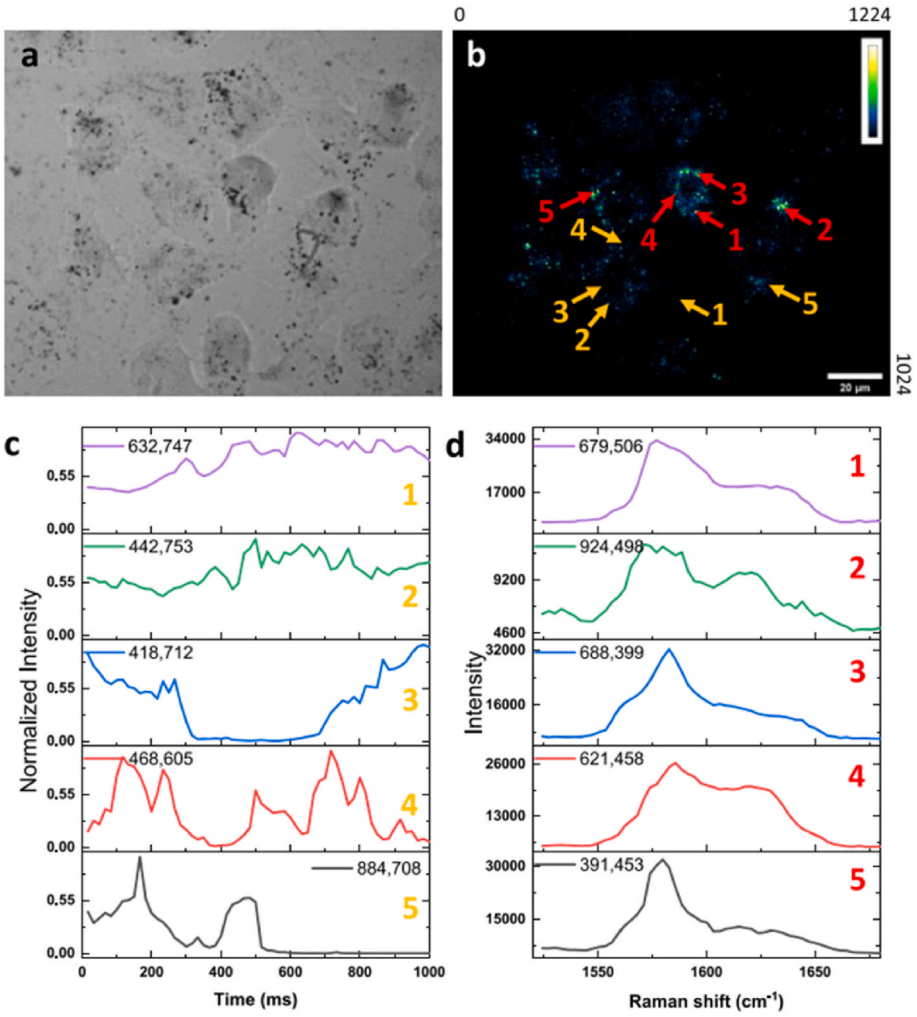

Fig. 9. Hyperspectral WISE-CARS imaging of live cells. (a) Bright-field image of T24 cells illuminated by LED. (b) Corresponding SECARS image of T24 cells at 1577 cm^{-1}. (c) Time-lapse curves of single clusters. (d) Single-pixel SECARS spectra of nanotags in live cells. The scale bar is 20 μm. Inside labels show the X–Y pixel coordinates where the spectra were recorded. Yellow arrows indicate where time-lapse curves are selected in the image. Red arrows show the locations where single-pixel spectra are selected. Reprinted with permission from [59]. Copyright 2022 American Chemical Society.

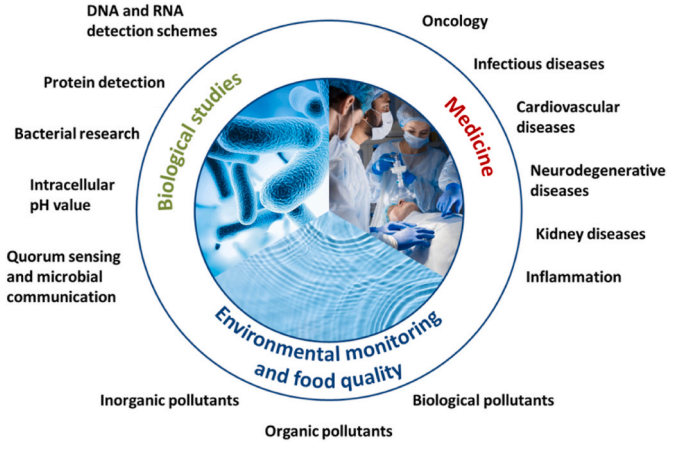

Fig. 10. SERS biosensing applications addressing the fields of medicine, environmental monitoring and assessment of the food quality as well as biological-driven studies.

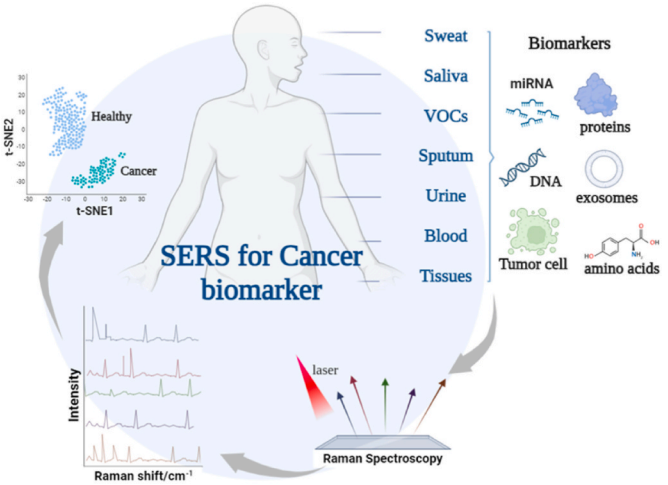

Fig. 11. Schematic illustration of SERS analysis workflows for cancer biomarkers detection in clinical diagnosis [62]. Reproduced under a Creative Commons license.

such as body fluids, it is crucial to implement multivariate data analyses. The use of multivariate methods helps in coping with the inter-individual normal variation underlying pathological changes, as detected by SERS. However, multivariate methods must be correctly validated; otherwise the results reported are likely to be overestimated.

Moreover, SERS holds tremendous potential in the field of tumor margin assessment, providing real-time and accurate information about the boundaries of cancerous tissues. Aiding surgeons in making informed decisions regarding tissue resection, SERS could revolutionize surgical procedures and improve patient outcomes. In a recent study, an intraoperative SERS navigation strategy delineating the acidic margins of glioma has been developed, enabling an evaluation of cancer tissue, i.e. estimation of the perimeter and size of the tumor site [73]. For ovarian and colon tumors resection, SERS-based pre-operative mapping and post-surgical adjuvant photothermal therapy have considerably improved the survival rate of mice when compared to the control ones [74]. SERS imaging-guided precision near infrared (NIR) photothermal anti-tumor therapy has been applied for colorectal cancer ablation *in vivo* [75]. This combined approach opens a promising way for in deep-tissue penetrating and single molecular resolution with high specificity. A similar double strategy, merged with chemotherapy, has been reported for the treatment of HeLa cancer cells [76]. Here, doxorubicin, a widely used chemotherapeutic drug, has been delivered in cancer cells nuclei thanks to the NIR-induced photothermal therapy, causing the death of the targeted cells. Consequently, the re-growing of the tumor will be suppressed and this approach does not induce damages to other organs.

Although therapeutic drug monitoring (TDM) has been sparsely utilized in oncology apart from some specific drugs such as methotrexate (MTX), there is an emerging body of evidence suggesting that it can be a useful tool for managing other chemotherapy drugs. Recently, SERS spectroscopy has established itself as a tangible new possibility for the sensitive, specific, and real-time monitoring of anticancer drug levels in biological samples, with some advanced evidence at least for MTX and 5-fluorouracil (5-FU). A nanopillar-assisted separation SERS-based detection method for quantifying MTX in the serum of leukemic pediatric patients receiving high-dose MTX therapy was developed [77,78]. When combined with an optimized sample pretreatment method (e.g. μ-SPE-SFH), SERS mapping, and chemometrics to reduce sample-to-sample spectral variability, the method reported a good LOD (5.7 μM) and LOQ (17.0 μM). While the complete clinical detection range (0.2–250 μM) is not yet fully accessible, identification of delayed MTX elimination is possible for a wide range of patients (Fig. 12). Moreover, a LFA-SERS technique to measure the concentration of 5-FU in actual blood samples, and the accuracy was verified using HPLC-MS/MS was utilized [79].

In summary, SERS-based detection has proved to be a powerful tool in oncology as cancer-related biomarkers are detected in a wide range of body fluids and patient samples. Thus, it is possible to differentiate between cancer patients and healthy volunteers based on the spectral fingerprint found via investigation of body fluids such as blood-based materials, urine or sputum. Moreover, tumor margins can be identified with SERS allowing for more precise surgery. In the case of cancer-related drugs, their detection in of high importance in the framework of TDM to allow for an improved monitoring of the patient to avoid severe toxic side effects due to too high concentrations of the drug molecule within the patient.

4.1.2. *SERS in infectious diseases*

4.1.2.1. Viruses and bacteria detection. Infectious diseases caused by viruses and bacteria can be identified using various biomarkers present in body fluids of sick people. These biomarkers are proteins of pathogenic membrane [80,81], proteins [24,31,82] and nucleic acids [83,84] from viral or bacterial lysates, and metabolites expressed by pathogens [49,85]. Both, direct (or label-free) and label-based SERS assays are successfully used for detection of such biomarkers. In the case of the direct format, the SERS signal can reveal a large amount of information from individual biomarkers, as well as from complex molecular assemblies and whole pathogenic particles. However, selective determination of infection biomarkers using direct SERS assays is challenging.

The first challenge is the low concentration of biomarkers in body fluids (especially in the early stages of the disease) compared to numerous endogenous components of the fluids (e.g., blood cells and proteins). Thus, culturing is often used to increase the concentration of pathogenic biomarkers before SERS-based detection [81,82,84]. However, this approach is time-consuming, and some researchers use filtration or ultrafiltration as a simpler alternative to concentrate biomarkers and separate them from the matrix [81,86]. For example, Dryden et al. [81] used membrane filters covered with AuNPs for simultaneous concentration and SERS detection of bacteria of urinary tract infection in urine. Infection biomarkers can also be trapped, separated and concentrated using magnetic NPs [83].

The second challenge is the realization of capturing biomarkers from the matrix and their immobilization at the SERS substrate. The simplest and less selective way to capture biomarkers is based on electrostatic interaction [85,87]. For example, positively charged gold nanorods possess strong interaction with negatively charged bacterial membranes [87]. Leong et al. [49] chemically functionalized SERS substrate with molecules of several thiolated Raman reporters which bind human

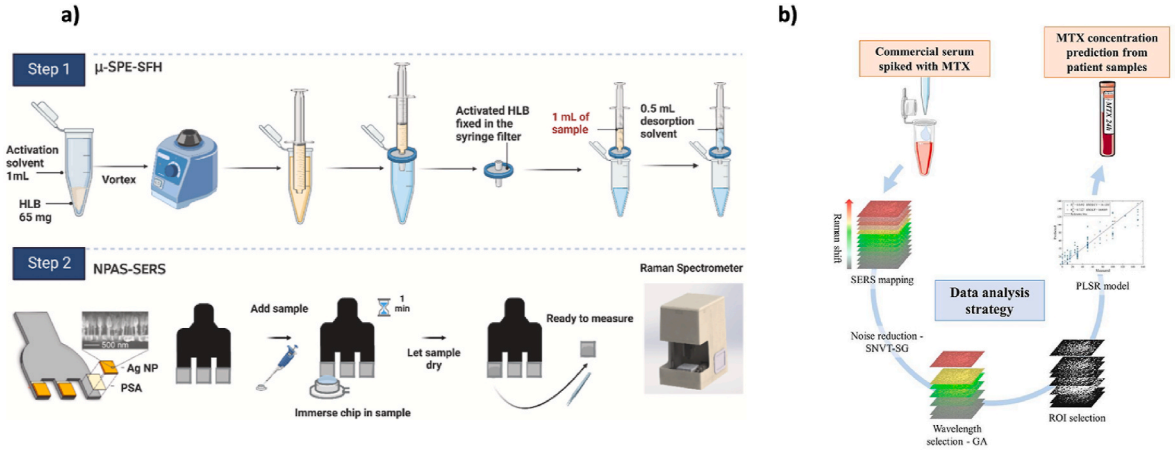

Fig. 12. a) Illustration of SPE-NPAS-SERS procedure. This procedure includes two steps, (i) preparation of the μ-SPE-SFH setup and extraction, (ii) NPAS and SERS analysis with Raman spectrometer [77]. b) data analysis strategy for SERS maps [78]. Reproduced from Refs. [77,78] under Creative Commons Attribution License 4.0 (CC BY).

breath volatile organic metabolites with change in SERS spectra of Raman reporters (Fig. 7). This approach was used to develop SERS-based breathalyzer for fast (5 min) and pretreatment-free diagnosis of COVID-19. In the case of bacteria, they can be captured through antibiotics that interact with the bacterial cell walls. Thus, various Gram-positive bacteria demonstrate significantly better adhesion to the SERS substrate coated with vancomycin [80]. Modification of the SERS substrate with bacteriophages also allows for capturing bacterial cells [88]. Electrokinetic techniques were also used to trap biomarker molecules before SERS analysis. For example, Chen et al. [89] separated and concentrated bacteria from human blood using dielectrophoresis. Lee et al. [82] applied EC-SERS approach, as introduced in chapter 3.4., for detection of SARS-CoV-2 in saliva samples by simultaneous electrodeposition of roughened Au layer and viral lysate at suitable potential (Fig. 13).

The third challenge is connected with the quite similar composition of bacterial or viral biomarkers. Thus, SERS spectra of biomarkers can have features from similar biomarker components such as amino acids, nucleobases, carbohydrates, etc. To overcome this challenge, almost all of the above works applied data analysis, which helps to identify biomarkers according to vibrational fingerprints and obtain reliable results. Moreover, data analysis also enables to discriminate between different infectious diseases [84,90] or different variants of one infectious disease [81,90].

Various label-based SERS formats with immunoreagents [20,24] and aptamers [29–31] were used to detect infection biomarkers (including multiplex detection). For example, Chen et al. [31] developed aptamer-based SERS assay ("on/off" format) to diagnose and distinguish SARS-CoV-2 and influenza A/H1N1 at the same time. Liu et al. [20] developed immunoreagent-based SERS-LFA ("sandwich" format, see chapter 3.3. for more information) for determination of serum antibodies (IgM and IgG) against SARS-CoV-2. The developed assay has significantly increased sensitivity (approximately 800-fold compared to colorimetric LFAs) and requires only dilution of patient serum samples as sample pretreatment. Finally, many label-based SERS assays for detection of infection biomarkers have been tested on a large number of clinical samples of body fluids [20,24,30].

It is important to note that the concentration of various biomarkers depends on the growth phase of bacteria and viruses and the stage of the disease [91]. Therefore, accurate determination of concentration of viruses or bacteria in body fluids is still difficult using both label-free and label-based SERS approaches. The assays described in this section are more suitable for qualitative or semi-quantitative evaluation of the presence of pathogens in the body that is often enough to diagnose and prevent the spread of infection.

4.1.2.2. Antibiotic susceptibility testing. Identification of bacterial pathogens with antimicrobial resistance is another important direction, and SERS has also been proposed for antibiotic susceptibility testing (SERS-AST). Particularly important direction is the determination of multidrug-resistant pathogens as they lead to serious infection diseases. The AST study includes addition of antibiotic molecules to a target bacterial culture and monitoring of the cells' response to the treatment by measuring the SERS signal. Currently, direct SERS sensing is dominating in SERS-AST studies due to simplicity and flexibility of realization and, most importantly, ability for registration of metabolic profiles suitable for further chemometric analysis [92].

Generally, SERS-AST aims to distinguish between viable (resistant) and nonviable (sensitive) bacterial cells by tracking changes in (i) the structure and composition of outermost cell membrane (cell walls) or (ii) the metabolic activity of the cells before and after the treatment with antibiotics. Importantly, because SERS is a short-range technique, it is capable of detecting only compounds in close proximity to the SERS-active surface. Therefore, changes in the cell walls can be relatively easily investigated with direct SERS after deposition of SERS-active metallic NPs onto the cell surface. For example, Pramanik et al. [93] performed *in situ* study of SERS signal of *Salmonella Typhi* and *Salmonella DT104* samples (both at 100 CFU mL^{-1}) during the treatment with Augmentin that is a mixture of a β-lactam antibiotic (amoxicillin) and a β-lactamase inhibitor (potassium clavulanate). The authors showed that the signal of the susceptible strain (*Salmonella Typhi*) decreased almost 10-times after 90 min treatment while the signal of the resistant strain (*Salmonella DT104*) remained unchanged. Control SEM measurements of the treated cells revealed that the signal decrease is mainly associated with degradation of cell walls.

The interior metabolic changes in susceptible bacterial cells, on the other hand, can be studied with SERS only for metabolites expressed in extracellular space [94] or for cell lysate (chemically, osmotically or mechanically disrupted cell culture) [92]. In this case, metabolic activity of the susceptible bacteria correlates with expression of biomarkers associated with the cellular stress (e.g., purine derivatives: adenine, hypoxanthine, etc.); detailed overview of both normal Raman and SERS vibrations for such biomarkers is given by Dina et al. [95].

As an example, Gukowsky and He [94] performed thorough SERS study of metabolite expression in extracellular matrix after treatment of *Escherichia coli* cells with different antibiotics. Interestingly, washing solutions obtained during cleaning bacterial cells from the growth media and antibiotic molecules have been found to be the most suitable for SERS-AST study. The authors explained this fact by a specific influence of nutrient-free environment on the expression of purine metabolites and the deviation in the expression degree can be used for differentiating susceptible and resistant cell lines. In the other example, Thrift et al. [92] proposed the use of metabolic profiling to identify resistant bacteria and developed the SERS-AST protocol based on measuring SERS signal of cell lysates with further analysis of spectral data using machine learning algorithms.

Besides the high progress in the field, some critical moments of SERS-AST should be discussed. For example, the ability of SERS to analyze tiny amounts of the sample allows performing the SERS-AST within a couple of hours and less (0.5 h[92]; 1.5[94], etc.). However, unfortunately, almost

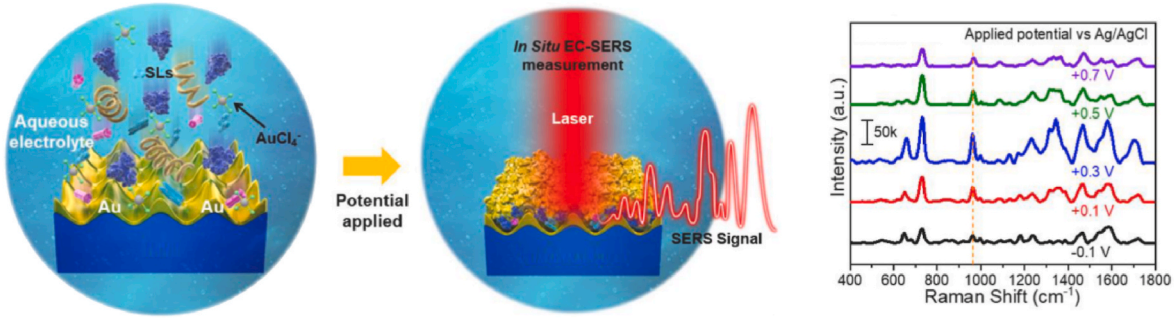

Fig. 13. Schematic illustration of EC-SERS detection of viral lysate (SLs) and influence of applied potential on SERS signal of viral lysate; adapted with permission from ref. [82].

all these studies anyway require preliminary time-consuming culturing step (≥24 h) in order to get enough bacteria cells for further SERS-AST. To overcome this limitation, electrokinetic preconcentration of bacterial cells [89] and cultivation of the cells in a microfluidic microwell device [96] have been proposed, reducing the total SERS-AST time to 2 h (including incubation step). Despite these achievements, additional studies are required to further improve the competitiveness of SERS-AST over standard culturing tests.

Since SERS-AST deals with bio objects it has some additional issues. First, such assays are label-free and sample pretreatment for *in vivo* studies is difficult to implement. For example, complete removal of the components of growth media before SERS measurement is mandatory during the study of metabolic activity, but even simple washing can influence cell metabolic response and AST results [94]. Second, antibacterial sensitivity of a given cell line can be affected by bacterial growth rate, temperature, and composition of the growth media (nutrient concentrations, ionic strength, pH). Moreover, the cells can express multiple purine metabolites which have good affinity to the SERS substrate surface and produce similar SERS spectra (particularly in the most used range of 720–740 cm^{-1}) [94]. Thus, direct and accurate interpretation of spectral results is very difficult and only application of multiple SERS peaks and multivariate spectral analysis enable to achieve required accuracy and speed of SERS-AST [92,97].

Additionally to direct detection methods, labeled SERS methods can also be utilized in drug susceptibility tests. As an example, labeled SERS was applied for bacterial detection as well as antibiotic susceptibility tests by constructing Au@Ag core-shell nanorods [98]. The SERS tags were equipped with R6G as reporter molecules, and for the recognition of *E. coli* cells, specific antibodies were used as modification. In the drug susceptibility testing, the authors detected the number of *E. coli* CFUs within an antibiotic culture, and the relation between the amount of *E. coli* and the SERS signal intensity was illustrated. The authors utilized the change of the SERS intensity to directly determine the inhibition and growth of bacteria to identify antibiotic resistance.

4.1.2.3. Therapeutic drug monitoring of antibiotics. TDM involves the quantification of drug concentrations circulating in body fluids to improve patient treatment. Thus, the implementation of TDM of antibacterial drugs into practice allows for optimizing the personalized dosing and reducing the antimicrobial resistance of bacteria. Antibiotic molecules usually have high polarizability and strong interaction with SERS substrates due to aromatic fragments and moieties with lone electron pairs ($–NH_2$ and $–COO^-$). Therefore, antibiotics generate quite intense SERS signal, and direct SERS assays were successfully developed and used for determination of antibiotics of different classes: cephalosporins [99], fluoroquinolones [100,101], sulfanilamides [102,103], etc.

The therapeutic concentration ranges of antibiotics in body fluids are approximately known from pharmacokinetic studies and these concentrations are relatively high (usually nM to μM levels) that reduces the requirements for sensitivity of SERS assays. However, endogenous components of body fluids may overlap and/or suppress SERS signal of antibiotics and different approaches were proposed to overcome such influence. For example, liquid-liquid extraction (LLE) [102] and solid phase extraction (SPE) [103] were used to separate analyte molecules from matrix before SERS-based determination of sulfanilamide antibiotics in urine. Chemical modification of SERS substrates can maximize the interaction of the SERS-active surface with analyte molecules and minimize the interaction with components of body fluids [101,104]. For example, Markina et al. [101] used β-cyclodectrins to modify AgNPs that increased the signal of fluoroquinolone antibiotics 5 times and suppressed background signals of urine and blood plasma. The correct choice of SERS substrate material is also important. For example, cephalosporin antibiotics showed the strongest affinity for the surface of copper NPs compared to AgNPs and AuNPs [99]. Also, cephalosporin molecules possess stronger interaction with the substrate compared to the molecules of urine components leading to the absence of background signal.

Importantly, the information about normal concentration ranges of body fluid components is also usually available and can be used to study and improve the assay selectivity. For example, selectivity and accuracy of some SERS assays were studied using urine samples with artificially increased concentrations of the main urine metabolites (e.g., creatinine, uric acid, electrolytes, etc.) simulating changes of urine composition at some diseases [99,101]. Additionally, SERS assays developed for antibiotics determination in other complex objects (e.g. food stuff) [104] can also be corrected and successfully adapted for analysis of body fluids.

Finally, SERS-based assays for TDM of antibiotics have several advantages compared to the assays currently used for this purpose. Compared to immunoassays, the SERS assays allow eliminating false-positive results thanks to the multiband nature of the SERS signal, and do not require quite unstable and expensive immunoreagents. Since antibiotics of the same class have quite similar chemical structure and properties, universal class-specific SERS assays were successfully developed [99,101] without the need to obtain antibodies for each specific antibiotic. Compared to HPLC-based analysis, SERS-based one is faster, less labor-consuming, and does not require specialized laboratories and trained personnel. For example, portable and easy-to-use Raman spectrometers were used in almost all mentioned above studies, demonstrating the ability of implementation of the proposed assays in hospital laboratories.

In summary, the main challenge of all works discussed above is the complex multicomponent composition of the analysed objects. Indeed, besides intrinsic components of body fluids from which the marker of a disease should be separated and identified, the pathogen itself is a complex multicomponent object with a certain structure (e.g., with bacterial/viral walls, internal contents, etc). Therefore, significant progress in these areas has been achieved due to the post-processing of spectral information by various chemometrics tools. Also, important to note that portable Raman spectrometers have been used in most of the work described in this section. Thus, SERS allows for performing three types of analysis of body fluids of patients with bacterial infections in hospitals: (i) detection and identification of bacterial infection, (ii) study of antimicrobial resistance of bacteria with AST in order to select the optimal antibiotic for the treatment, and (iii) performing TDM to achieve the most effective and safe treatment process.

4.1.3. Cardiovascular diseases

Most of recent SERS studies [105–107] on biological samples for diagnosis or prognosis of cardiovascular diseases focus on the indirect targeted analysis of various protein markers (e.g. cardiac troponins) in serum, aiming at the detection of acute myocardial infarction. Although in all studies the approach adopted is that of the SERS immunoassay, the details about how the analysis is carried out are different. Tu et al. use a LFA, as introduced in chapter 2.3. as powerful SERS platform for the point-of-care, to quantify a panel of three makers (i.e. cardiac troponin I, copeptin, and heart-type fatty acid-binding protein), by using three different SERS tags constituted by core-shell AuNPs with embedded reporters [106]. The approach proved successful in quantifying the markers at clinically relevant ranges in spiked human serum, even though some cross-reactivity emerged in the multiplexed assay. Lim et al. pursued a multiplexed SERS detection of other three cardiac protein markers (i.e. glycogen phosphorylase isoenzyme BB, troponin T and creatine kinase-MB) on a microfluidic paper-based device with multi-reaction zones, by using different SERS tags and antibodies as recognition elements [107]. Details on microfluidic SERS platforms can be found in chapter 2.1. This strategy proved successful for a multiplexed quantification of the markers in human serum at clinically relevant concentrations. A different strategy was applied in the work by Liu et al., in which two protein markers (i.e. troponin I and creatine

kinase-MB) were detected by trapping them in between a magnetic nanoparticle and a SERS tags using antibodies as recognition elements [105]. Both these nanoparticles were quickly mixed with human serum spiked with the markers to form complexes, and subsequently captured by a small magnet, on an integrated single-track finger-pump PDMS microfluidic chip as depicted in Fig. 14, allowing a simultaneous SERS quantification of the two targets at the clinical threshold values in few minutes.

Beside these works aiming at diagnosis, SERS has been also applied by Hua et al. to study stem cell therapy for myocardial infarction, by tracking stem cells in animal tissues with a dual-modal SERS/fluorescence imaging approach [108]. Nanoparticles formed by Au nanostars with a Raman reporter, subsequently covered with fluorescent Ag_2S quantum dots and antibodies specific for stem cells, were used to label the stem cells. Labeled stem cells were then transplanted in mice with induced infarction, and could be tracked in heart tissues with a high-resolution Raman imaging.

4.1.4. Neurodegenerative diseases

Neurodegenerative diseases are characterized by long-term cognitive decline, loss of memory, and functional decline. These symptoms are often not evident even after decades of neurodegenerative processes have taken place. Recent SERS studies focus on the early detection of both characteristic biomarkers for one disease (e.g. specific amyloid-β proteins, Aβ, in Alzheimer disease) and general biomarkers for neurodegeneration, which correlate with structural and functional changes in brain tissues and neurons, indicating abnormal biological and pathological processes in-stage. In the case of Alzheimer disease (AD), Yang et al. developed a SERS immunosensor using head-flocked nanopillar arrays and SERS nanotags modified with half antibody fragments for quantitative determination of tau protein in the plasma of AD patients [109]. The sensor showed a wide detection range (10 fM – 1 μM), and a LOD of 3.21 fM. Wang et al. used a direct SERS approach involving chiral plasmonic triangular nanorings to assess the secondary structure of 42-residue-long amyloid-β proteins [110]. The method was applied to cerebrospinal fluid (CSF) samples of Alzheimer patients, showing potential in identifying diseases based on protein-misfolding. A very interesting new approach was described in a proof-of-concept study by D'andrea et al., in which the seed amplification assay technique was combined with SERS, to detect the presence of pathological amyloid-β species (typically found in the brain) in the CSF of patients with a clinical diagnosis of AD [111].

General neurological biomarkers such as matrix metalloproteinase (MMP-9) [112],soluble platelet-derived growth factor receptor, S100 calcium-binding protein B protein (S100-β) [113], Neuro-specific enolase, and major facilitator superfamily domain containing Mfsd2a, were detected in blood and CSF using various SERS-based approaches, with detection limits as low as 1 fg mL^{-1} [114]. The increasing role of neurotransmitters in neurological, endocrinological, and immunological processes has led to the increased use of NTs as a main focus for the development of biomarkers that can predict or correlate with nervous system function. In this context, prior studies have documented the use of SERS strategies to detect various NTs, such as dopamine, melatonin, serotonin, γ-aminobutyric acid, and acetylcholine [115]. More recently, quantitative SERS detection of dopamine was reported in human serum using both direct and indirect detection schemes, with detection limits reaching the aM and pM range, respectively [116,117].

4.1.5. Kidney diseases

SERS-based renal function evaluation has recently been reported for chronic kidney disease (CKD), acute kidney injury (AKI), and for rapid assessment of kidney transplant function. Zong et al. compared the differences between the SERS spectra from serum and from 126 CKD patients (Stages 2–5) and 97 healthy individuals using a simple untargeted direct approach with hydroxylamine-reduced silver nanoparticles and principal component analysis combined with linear discriminant analysis (PCA-LDA) [118]. Correlations between of kidney functional parameters and SERS spectra from serum or urine were also reported, showing high correlation for serum urea, SCr and eGFR, urinary urea and urinary creatinine. Similarly, in a study involving 110 kidney transplant recipients, Huang et al. found high correlation between SERS spectral signatures with urinary and serum creatinine [119]. The levels of serum creatinine and blood urea nitrogen (BUN) are the most widely used biomarkers for kidney biofunction, and quantitative SERS detection in serum have also been proposed. Yang et al. reported a SERS chip integrated with a micro-optical system (comparable platforms are described in chapters 2.2. and 2.3.) with a detection limit for creatinine in serum of 5 μM [120]. This method was also used to discriminate spectra of normal human serum and that of patients with renal dysfunction. In another study, a CNN-assisted SERS detection scheme for serum creatinine and BUN was also introduced using Au core-Ag shell nanoprobes [121]. Various conditions were simulated by mixing both biomarkers in varying concentration in fetal bovine serum and a prediction of the concentration of both target analytes was achieved in

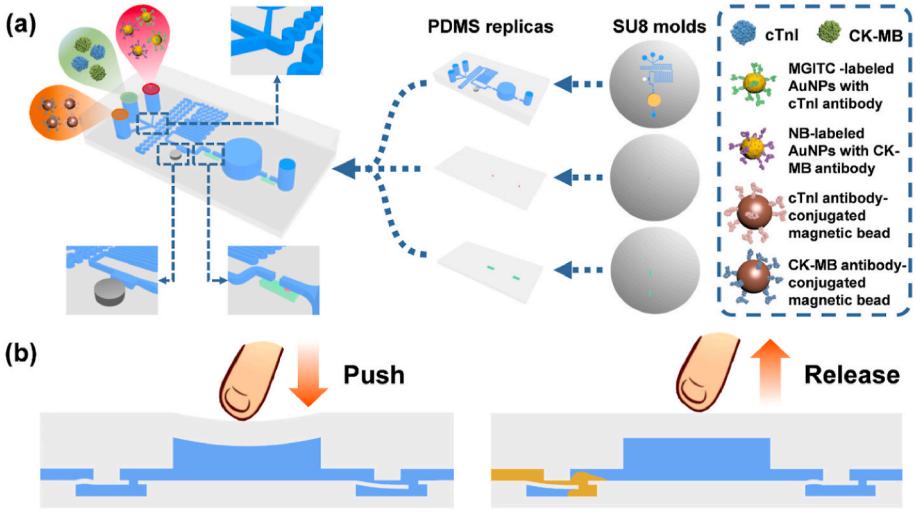

Fig. 14. (a) The work-flow of the SERS-based finger-pump microfluidic chip for simultaneous detection of dual acute myocardial infarction biomarkers. Three reagents were dropped at the inlets of the chip. The fabrication process of the three-layer finger-pump microfluidic chip was shown in the right-hand. (b) Lateral view of the finger-pump microfluidic chip, and the schematic representation of check valves when the finger-pump was pressed and released by fingers. Reproduced with permission from [105].

spiked serum samples.

4.1.6. Inflammation

Inflammatory state (local or systemic) is present in many illnesses. Recent SERS applications, however, are mostly focused on characterizing and detecting a systemic inflammation associated with infection, and in particular on sepsis, for diagnostic purposes. Many authors pursued a targeted indirect SERS analysis of inflammatory protein markers (e.g. CRP, interleukin-6 and procalcitonin) by using different SERS tags. A SERS immunoassay approach was adopted: with a LFA system [50], and using a vertical flow based on a nanoporous anodic aluminium oxide membrane capable of quantifying up to four different markers in spiked serum at clinically relevant concentrations, as illustrated in Fig. 15 [122]. Zhou et al., conversely, rely on a "sandwich" approach in which targets are bound, via aptamers, in between two different SERS tags: an Ag-coated Fe_3O_4 magnetic nanoparticle labeled with an SERS internal standard, and Au–Ag urchin core/Au porous shell nanoparticles modified with Raman reporters [123]. Such nanoparticles assemblies could be magnetically separated and detected with SERS, allowing the detection of three protein markers in human serum at clinically relevant concentrations. In general, SERS strategies based on indirect targeted detection, despite the differences concerning the structure and characteristics of the SERS tags used and of the coupling and separation methods, proved to be potentially relevant in clinics by quantifying inflammatory protein markers to diagnose severe infections.

Untargeted SERS approached are also possible: Fornasaro et al. used an untargeted SERS analysis of gingival crevicular fluid (GCF) to compare absence and presence of inflammation in periodontal tissues [124]. The analysis reported that SERS spectra of GCF are very similar to those found in the case of deproteinized serum, and samples from inflamed tissues yielded SERS spectra with differences with respect to those of healthy tissues. In particular, those spectral differences could be interpreted in terms of some metabolites, with inflamed tissues having a lower relative amount of ergothioneine with respect to healthy ones.

4.1.7. Other diseases

Diabetes diagnosis or management has been the subject of some recent SERS studies. Kim et al. developed a SERS immunoassay protocol to indirectly quantify adiponectin in serum for the diagnosis of gestational diabetes [125]. In particular, the target was captured by using antibodies in between an Au surface and Au nanotriangles conjugated with a Raman reporter, in a characteristic "sandwich" SERS detection strategy. A sandwich detection strategy was also used by Zhang et al., on the other hand, aiming at glucose sensing with SERS, a topic widely explored in the past by several authors [126]. In particular, this promising study selectively captured the target by forming *cis*-diol compounds between glucose and 3-aminopheyonul boronic acid (APBA) linked to AgNPs. A second nanoparticle, conjugated both APBA and a Raman reporter with a triple bond, allowed the indirect detection of glucose in a "silent" region of the spectrum (at 1977 cm^{-1}), achieving glucose detection at 10^{-11} M in artificial urine.

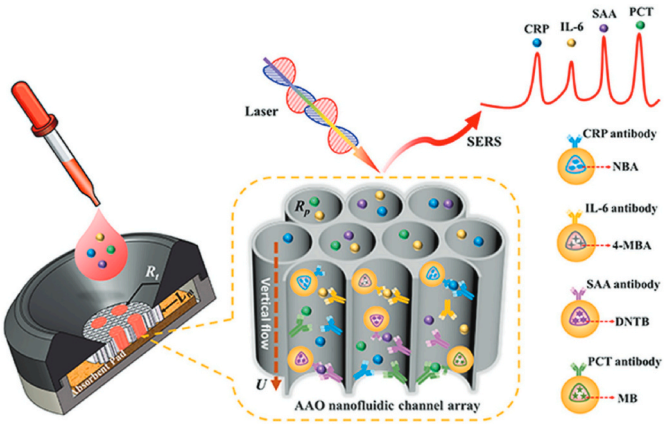

Fig. 15. Schematic illustration of nanoporous AAO-based multiplex vertical flow assay (VFA) for the detection of four inflammatory biomarkers with Raman dyes encoded core–shell SERS nanotags. Characteristic Raman peaks of NBA at 593 cm^{-1}, 4-MBA at 1075 cm^{-1}, DNTB at 1341 cm^{-1}, and MB at 1621 cm^{-1}, are used to encode CRP, IL-6, SAA, and PCT, respectively. Reproduced with permission from [122].

Among other recent interesting SERS sensing applications, the direct targeted detection of clinically relevant metabolites in sweat was introduced. Kim et al. quantified both creatinine and cortisol, two markers for various diseases, in human sweat with SERS by using Ag nanosnowflakes deposited on hydrophobic paper substrates [14]. Mogera et al. successfully and selectively quantified uric acid, another metabolite proposed as marker for several pathologies, in human sweat with SERS by using an innovative wearable plasmonic paper-based microfluidic system, as shown in Fig. 16 [19]. The wearable sensor proposed is very stable under various, and it does not need calibration. Other examples of microfluidic SERS platforms and portable SERS devices are previously introduced in chapters 2.1. and 2.3.

Conversely, indirect targeted SERS detection schemes of metabolic or protein markers in biofluids by monitoring the spectral changes due to chemical or conformational variations or a reporter upon target binding were developed. In particular, Kao et al. evaluated the risk of miscarriage upon indirect quantification of two metabolites (i.e. pregnane and tetrahydrocortisone) in urine by SERS [18]. Superhydrophobic Ag susbtrates were grafted with 4-mercaptophenylboronic acid, whose SERS spectrum changed upon selectively binding the targets by forming of a boronate ester bond. Substrate hydrophobicity was instrumental in concentrating the sample, further enhancing the SERS signal and enabling the multiplexed detection of these metabolites at sub-nanomolar concentrations in 30 min. A similar detection strategy was adopted in the work by Zheng et al., in which thyroid-stimulating hormone (TSH), a protein marker for thyroid functional disorders, was quantified in serum by detecting spectral variations due to structural changes of 4-mercaptobenzoic acid (4-MBA), conjugated to a TSH antibody, upon binding of a TSH antigen [17]. The band at 1580 cm^{-1} of the 4-MBA is due to a vibrational mode of its benzene ring, and it shows a small but detectable shift upon nanomechanical perturbation, such as target binding to the linked antibody. The method proposed, which included the setting up of a partial-least-square prediction model and its validation, successfully quantified TSH in serum samples at clinically relevant concentrations.

In summary, SERS literature seems to mostly focus on those pathologies related to an aging population, such as cardiovascular, neurodegenerative and metabolic diseases. Most often than not, protein markers are the target of the analysis, which is then pursued via indirect SERS detection strategies. Lateral flow and microfluidics are used in combination with SERS tags in many studies, with the aim of making sample pre-treatment and liquid handling as easy and compact as possible. A multiplexed later-flow SERS immunoassay approach to detect protein markers, in direct competition with ELISA tests, seems to be the trend for the diagnosis and management of the pathologies listed above.

4.2. SERS in environmental monitoring and food quality control

The main requirement for SERS in the case of food and environmental analysis is high sensitivity because concentrations of pollutants are mainly at ppb level (μg kg^{-1}, μg L^{-1}). The sensitivity can be achieved by the development of new SERS substrates with increased enhancement factor and/or sample pre-concentration before SERS determination. The concentrations of pollutants are usually indicated in quality directives, and the protocols for their determination have to be optimized to the required level of sensitivity. Regardless of the analytical method, a major challenge in determining contaminants in food and the

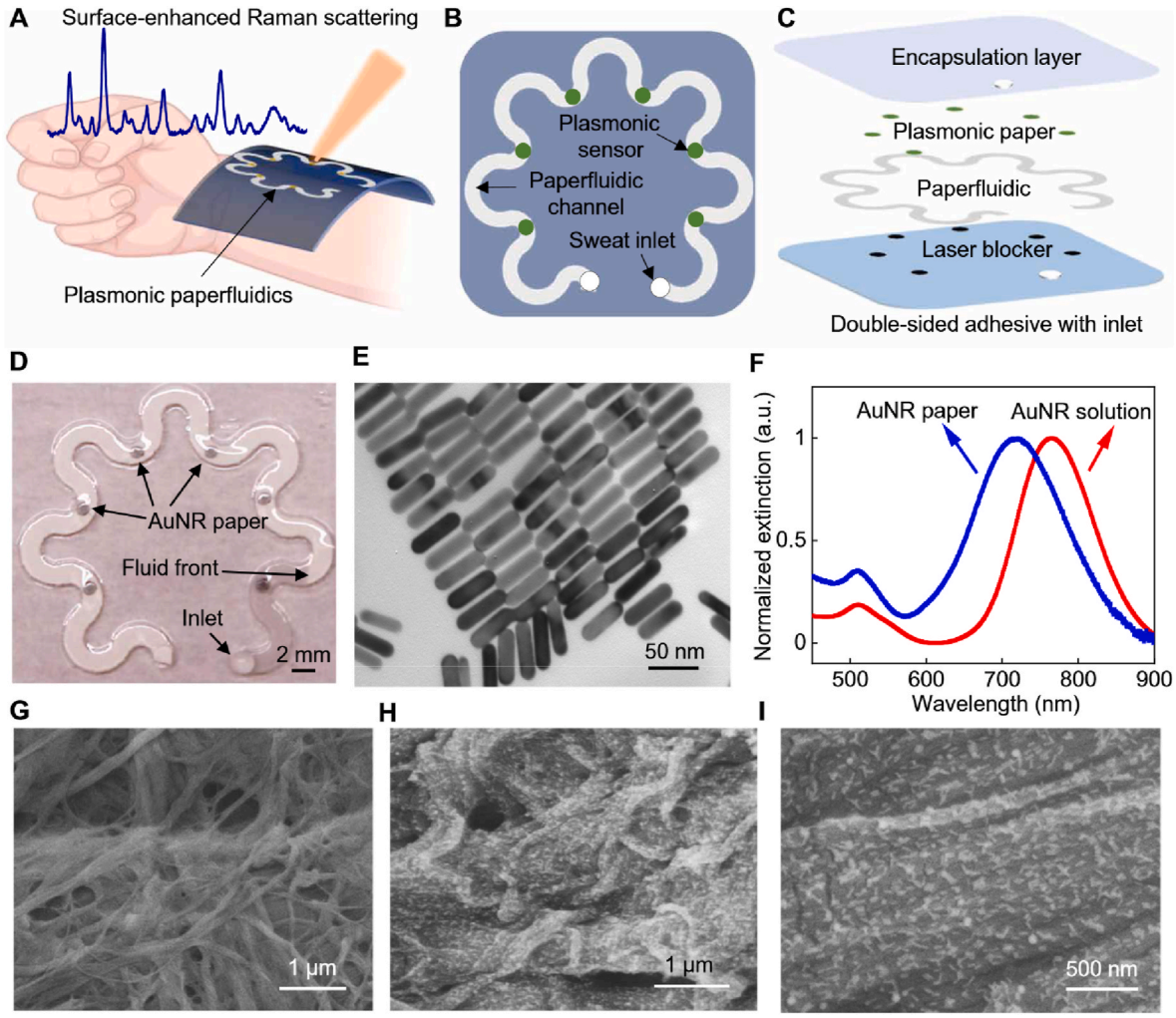

Fig. 16. Wearable plasmonic paper microfluidic device. (A) Conceptual illustration of a wearable plasmonic paperfluidic device for sweat collection, storage, and *in situ* analysis using SERS. (B) Top view and (C) stacked view schematics of the paperfluidic device highlighting key functional layers. (D) Photograph of an assembled paperfluidic device with six plasmonic sensors. (E) TEM image of gold nanorods (AuNRs) with a uniform size distribution. (F) Extinction spectra of AuNR solution and AuNR paper. a.u., arbitrary units. SEM images of pristine chromatography paper (G) and AuNR paper (H and I). Reproduced from Ref. [19] under Creative Commons Attribution License 4.0 (CC BY).

environment is the complexity and diversity of the matrix composition. Therefore, works dedicated to the analysis of pollutants in samples with simple matrices will not be discussed in this section (e.g., drinking water or swabbing from peel of vegetables and fruits), since such objects are not challenging for modern SERS analysis. The pollutants can be classified as inorganic (heavy metals [127–129], nitrite-ions [130], etc.), organic (pharmaceuticals [131,132], pesticides [133–135], etc.) and biological (bacteria [23,28], mycotoxins [25,35,136], biogenic amines [137], etc.).

4.2.1. Inorganic pollutants

Ions including several atoms (e.g., nitrite) produce too weak SERS signal in order to be suitable for direct analysis. Therefore, determination of such pollutants can be done only indirectly and using additional reactions. For example, Zhang et al. [130] proposed to use the azo reaction between two 4-aminothiophenol (4-ATP) molecules adsorbed on the AgNP surface in the presence of nitrite ions to determine these ions in sausages and pork (Fig. 17a).

Heavy metal ions do not have characteristic vibrational Raman peaks at all and consequently, cannot be detected by SERS directly. Thus, an effective strategy is the formation of coordination compounds with Raman reporter molecules. For example, Chen et al. [127] used an organic ligand with intense SERS signal (trithiocyanuric acid) as a stabilizer for AgNPs to develop a protocol for Cd^{2+} determination in black tea. In this assay an increase in the Cd^{2+} concentration in a sample leads to an increase in the SERS signal due to the formation of the "Cd^{2+}–trithiocyanuric acid" complex. Unfortunately, an important limitation of such assays is that the same ligands can also capture cations of other metals. To overcome this limitation, Song et al. [128] proposed pre-separation of Cu^{2+}, Cd^{2+}, and Ni^{2+} in rice sample using paper chromatography. Filter paper coated with AuNPs and modified with 4-MBA was used as a stationary phase, and solution of AuNPs@4-MBA was also added after chromatography to the zones with separated ions (Fig. 17b). In this case, the SERS signal of 4-MBA was additionally enhanced in the presence of metal ions due to the formation of a coordination compound with "sandwich" structure: metal ions are captured between AuNP@4-MBA in the stationary phase and added after separation.

Another strategy was proposed for determination of Hg^{2+} which is based on ability of these ions to react with metallic NPs forming amalgams. For example, addition of Hg^{2+} to the mixture of Au@AgNPs and Raman reporter (R6G) results in a decrease of the R6G signal due to formation of the Ag–Hg amalgam and desorption of R6G molecules from the silver surface [129]. This approach was successfully used for selective determination of Hg^{2+} in fish.

Importantly, heavy metal ions are capable to form complexes with

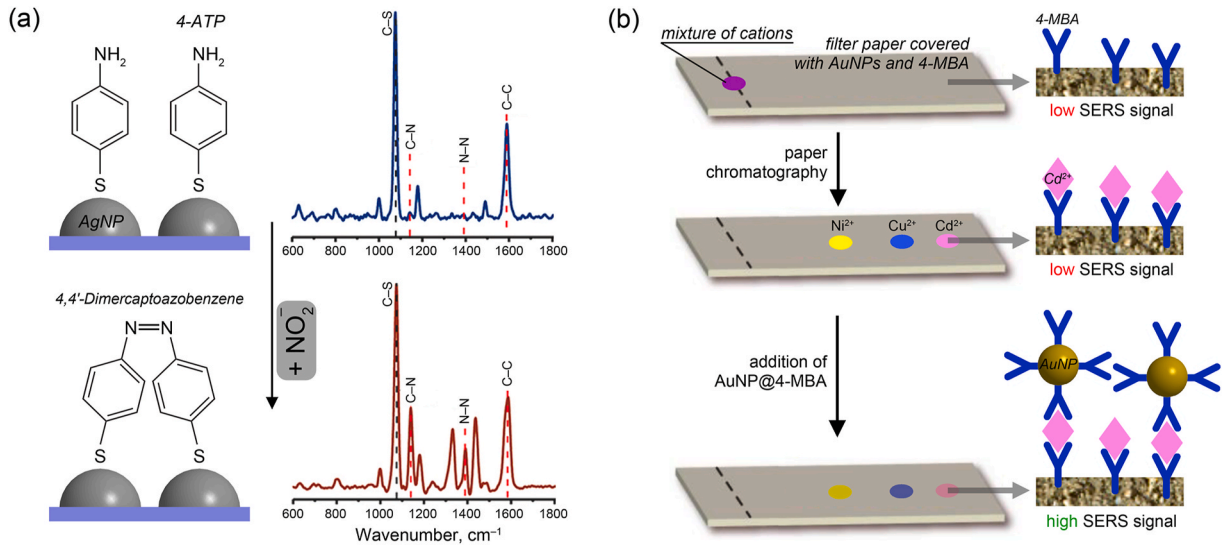

Fig. 17. (**a**) Schematic illustration of SERS detection of nitrite ions based on azo reaction between 4-aminothiophenol (4-ATP) molecules attached to the surface of AgNPs in the presence of nitrite ions; adapted with permission from Ref. [130] (**b**) Schematic illustration of SERS detection of heavy metal ions based on paper chromatography separation and formation of SERS-active coordination compound; adapted with permission from ref. [128]. Copyright 2020 American Chemical Society.

intrinsic food components, e.g., proteins [129] or tannins [127]. Thus, the protocols for their determination mainly include the digestion stage that includes oxidation of organic substances with concentrated nitric acid. However, this step of sample pretreatment is also mandatory for determination of heavy metal ions using other methods (e.g., ICP-MS).

4.2.2. *Organic pollutants*

SERS assays for determination of pharmaceuticals and pesticides represent the largest part of the articles devoted to the SERS-based monitoring of environmental pollution and food quality control. This fact is explained by intrinsically high polarizability of such organic molecules and, therefore, high intensity of their SERS signals. Most of the SERS assays used for determination of these pollutants are direct. As a result, separation of target analytes from the object matrix before recording SERS spectra is usually a mandatory step, and LLE [132,134] and SPE [133] were used for this purpose. In recent years many SERS assays for pollutant detection have used a sample pretreatment called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) [132,135]. However, this technique is actually quite complex and time-consuming and includes both LLE with acetonitrile and dispersive SPE. Moreover, the extracted analyte molecules have to be transferred from organic solvent to water in order to eliminate strong background Raman signal from the solvent. Therefore, although this technique has been claimed to be universal, this claim may be considered as correct only for methods, for which it was originally developed – chromatography. Thus, from our point of view, the efficiency and universality of QuEChERS in SERS analysis are overestimated and this technique should be carefully and critically used for development of new SERS assays.

It should be noted that some studies have shown that the object matrix can affect the signal intensity of the analyte even after sample pretreatment [132,133]. For example, calibration plots for determination of thiram in different juices (pear, apple, and orange) have different slope values [133]. This fact means that the proposed pretreatment is not universal enough and the same analysis protocols for determination of the same analyte cannot be extrapolated to the analysis of other objects of the same foodstuff type. Multiplex determination of organic pollutants was achieved using thin-layer chromatography (TLC) for sample pretreatment before SERS detection. For example, Shi et al. [131] developed a TLC-SERS protocol suitable for simultaneous determination of 14 veterinary drugs in minced pork using standard TLC plates and citrate-stabilized AuNPs. Such good universality has been achieved due to careful optimization of separation conditions and the use of chemometrics for analysis of SERS signal of poorly resolved analytes.

Another type of organic pollutant, which should be discussed, is microplastics (MP). Proposed direct SERS protocols involve (i) coating of planar SERS substrate with MP or (ii) mixing of colloidal SERS substrate with MP and drying the mixture. Detection of MP particles is carried out by mapping that requires stationary and expensive Raman spectrometers capable of mapping with high spatial resolution. The works show the possibility of successful identification of plastic type by characteristic SERS peaks [138–140]. However, determination of MP particle concentration with SERS is impossible because in this case the intensity of SERS signal of MP particles simultaneously depends on concentration and size of the particles [138]. Therefore, SERS is used only for identification of MP material, and the size and concentration of MP particles are determined using nanoparticle tracking analysis [140] or SEM [139].

4.2.3. *Biological pollutants*

For SERS detection of pathogenic bacteria the most reasonable way is to use a label-based SERS approach. Cell walls of different bacterial species consist of the same basic biological components, and label-free SERS approach usually cannot distinguish between multiple bacterial species within the same sample. Both antibodies [23] and aptamers [20] were successfully applied as recognition elements for “sandwich” format analysis allowing to perform simultaneous determination of two pathogenic bacteria in milk [23] and different seafood samples (salmon, oysters and shrimp) [28]. Importantly, both assays demonstrated high sensitivity, despite simple spherical AuNPs were used to prepare the SERS tags.

Mycotoxins are toxic metabolites produced by fungi, and for their determination both direct and label-based formats have been proposed. Zhu et al. [35] used gold-based SERS substrate modified with MIP for the determination of patulin in jam and juice samples. The authors showed that this MIP-SERS substrate better adsorbs and enhances the Raman signal of patulin compared to its structural analogues (oxindole and 5-hydroxymethylfurfural). However, the assay has quite poor selectivity and accuracy because the intensity of patulin spectrum decreases by ~25 % upon detection of patulin in an equimolar mixture with both

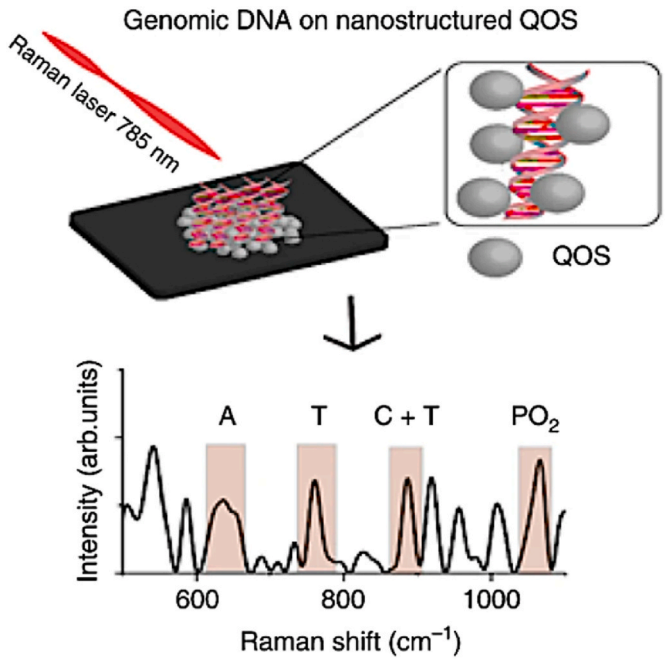

Fig. 18. Schematic illustration of DNA base composition analyses [143]. Reproduced under a Creative Commons Attribution 4.0 International License.

structural analogues. To achieve accurate SERS-based determination of ochratoxin A in wine and wheat, Rojas et al. [136] applied preliminary analyte separation from background-forming components using carefully optimized LLE. Finally, Zhang et al. [25] developed a SERS-based LFA for the simultaneous determination of 6 mycotoxins in maize. Such good performance was achieved due to the use of SERS tags for each mycotoxin (two Raman reporters were used) and three test zones for signal registration.

The food quality control also involves SERS monitoring of biogenic amines that is used to identify microbial spoilage of foodstuff. For example, Filipec et al. [137] carried out SERS-based determination of histamine in fish and used 2 sets of fish samples in the study: calibration and real. The first one included fresh fish spiked with known histamine concentrations. The second one included fish stored at room temperature that led to its contamination with histamine due to natural microbial spoilage. There were also 2 types of fish in each set: 7 samples of bluefin tuna and 5 samples of bonito. Histamine was extracted from fish muscle using LLE, and citrate-stabilized AgNPs were used as the SERS substrate. First, the influence of the fish type on the rate of histamine formation in the set with real samples was demonstrated. Second, samples with naturally produced histamine have more complex and intense background signals compared to samples of the calibration set due to decomposition of the sample matrix during spoilage. To overcome issues with matrix effect and correctly predict histamine concentration in fish using SERS, the authors investigated various chemometric tools and the best results have been achieved using artificial neural networks in combination with principal component analysis (PCA).

In summary, the analysis of publications showed that objects of varying complexity were used as matrices during the development of SERS assays (solid/liquid, protein- and sugar containing, etc.). However, frequently observed disadvantage of the assay testing is the use of only a single sample of the object (e.g., one apple, one juice, etc.). Thus, the influence of possible variability of the matrix composition on the analysis results has been rarely tested. On the other hand, studies performed in this direction clearly demonstrate the appearance of such influence when moving from one object to another [133,137]. Thus, expanding the number of test objects in order to find a specific matrix effect (e.g., determination of pesticides in different types of tea: black, green and herbal) is critically important practice for development of versatile and accurate SERS assays for environmental and food analysis. It should also be noted that sample pretreatment in SERS is often comparable in complexity to sample pretreatment in HPLC. In some cases such pretreatment is mandatory (e.g., for determination of metal ions in foodstuff), while simplification and speeding up the pretreatment for on-site analysis is a promising direction. For example, improvement of selectivity, sensitivity, and accuracy can be achieved by modification of the SERS substrate surface (e.g., with anti-foiling or MIP coatings). Finally, the key challenge of environmental and food analysis is that the type and amount of pollutants (i.e., analytes) are unknown. However, there is a lack of papers where the SERS identification of unknown analytes in a sample has been demonstrated (even using databases of SERS spectra). Moreover, the SERS protocols are mainly developed for one specific analyte or several analytes of the same group. Therefore, the development of multiplex SERS assays suitable for simultaneous determination of several analytes and identification of unknown pollutants are two critically important directions.

4.3. SERS in biological studies

SERS is a promising spectroscopic analytical method in biological applications, especially in terms of medical research and diagnostics, including bacteria and their resistance pattern and cancer cells, biomarkers and genomic material. It can provide reliable information about changes in DNA structure, information about protein structure, and amounts of pathogens or bacteria in complex matrices, simple systems such as low molecular weight nucleic acids and proteins, or complex systems such as cells or tissues. Within the following chapter, SERS-based applications in biological studies are focused.

4.3.1. SERS-based DNA and RNA detection schemes

As a technique, SERS can identify different DNA and RNA sequences due to its sensitivity and fingerprinting ability, making it useful in fields such as genomics, biosensing, and forensic applications. SERS can identify DNA conformational changes, mismatches, and hybridization events as well as methylation levels and point mutations.

As an example, a sensor concept utilizes a microfluidic platform to directly identify circulating tumor DNA (ctDNA) in whole blood [141]. The system employs a novel approach that negates the need for initial sample processing. A microfluidic filter trench is used to effectively separate cells from the blood, and the purified sample is then channelled to a SERS substrate for mutation detection. The substrate made of gold@silver nanorods significantly enhances the sensitivity of detection. The sensor has successfully detected the EGFR E746-A750 mutation, commonly associated with lung cancer, at concentrations as low as 100 fM in whole blood during clinical applications. This technology empowers the use of cell-free DNA fragments to monitor tumor dynamics, therapy responses, and the emergence of drug resistance in real-time. The streamlined process, from sample introduction to SERS signal readout, takes 35 min. This marks a significant advancement in point-of-care molecular diagnostics, providing a rapid and dependable tool for managing clinical treatment and monitoring. The sensitive detection of ssDNA at the 100 nM level for mecA and intI1 gene pathogens was achieved using the slippery liquid-infused porous SERS (SLIPSERS) method [142]. The evaporation of a water droplet containing gold nanoparticles and ssDNA generates a highly sensitive hot spot on the SLIP membrane. A machine learning-based model was successfully used to discriminate between gene sequences. The model was able to distinguish SERS spectra of 12 gene sequences with mecA, intI1, and their analogues with 2–10 base differences and two random sequences. The model achieved an accuracy of 90 % in identifying each gene sequence, with sensitivity down to a single base. The direct SERS detection of single-stranded DNA related with gene segments responsible for antibiotic resistance against methicillin (i.e. mecA) and B-lactam antibiotics (i.e. intI1) shows the potential for clinical research.

Epigenetic DNA alterations, such as methylation, base composition, and gene expression, are crucial in identifying therapeutic targets. A study uses organic semiconductor SERS probes for direct genomic DNA detection (Fig. 18), highlighting enhanced charge mobility for efficient substrate-analyte charge transfer [143]. Quantum Organic Semiconductor (QOS) probes were synthesized with quantum-scale precision using ultrashort pulsed lasers. Validation was performed on various cell lines, such as NIH3T3 fibroblasts and MDA-MB 231, AsPc-1, and H69-AR cancer cells. The results showed accurate detection of DNA features in a single assay. Multivariate analysis was employed to differentiate molecular differences between cancerous and non-cancerous genomic DNA. This provides a more precise approach for epigenetically informed cancer treatment strategies.

A sensitive biosensor was presented that incorporates catalytic hairpin assembly (CHA) to detect miRNA-21 and miRNA-155 [144]. The biosensor uses an innovative Au–Si 2D substrate in combination with Ag@4-MBA@Au core-shell nanoparticles, achieving detection limits as low as 0.398 fM and 0.215 fM, respectively. The integration of RNA recycling amplification and SERS hot spots significantly enhances detection. This method is highly effective in distinguishing healthy serum from serum affected by breast cancer, highlighting its potential. It provides a promising avenue for early diagnosis and monitoring. Finally, a deep learning integrated SERS sensor rapidly detects RNA in human nasopharyngeal swab samples [145]. Using a silver nanorod array functionalized with a DNA probe, the sensor accurately identifies viral RNA, achieving a detection range of 10^3 to 10^9 copies/mL. A deep learning model further enhances its accuracy, delivering 97.2 % accuracy in blind tests. The entire process takes approximately 25 min, demonstrating its potential as a rapid and reliable diagnostic tool for SARS-CoV-2.

In summary, SERS accurately detects DNA changes that are critical for the diagnosis and monitoring of diseases. The integration of SERS with machine and deep learning algorithms enhances its application in clinical diagnostics, effectively identifying DNA abnormalities.

4.3.2. Protein detection

Proteins can be detected by both direct and indirect SERS methods. Although, direct SERS is limited by its ability to detect only proteins with significant affinity for plasmonic metals and requires rigorous sample preparation. In contrast, the indirect method, which uses antibodies for targeted protein capture, is proving to be a highly selective technique that is attracting considerable attention.

Many proteins are characterised by the presence of disulfide bonds in their structure. Human insulin, which is essential for regulating blood sugar levels, contains such bonds. A direct SERS method is developed for the sensitive detection of insulin [146]. This method uses antibody-functionalized magnetic nanoparticles to extract insulin from blood plasma, converting the disulfide bond to a thiol (-SH) group for stable chemisorption on a gold-coated copper oxide substrate. This approach allows a uniform distribution over the hotspot regions, achieving detection limits as low as 10 zM with a relative standard deviation of 5.52 %. The efficacy of this SERS-based detection is further validated by cross-referencing with enzyme-linked immunosorbent assay (ELISA) results.

As a further example, a highly sensitive technique for the detection of proteins in serum samples was presented [147]. Protein extraction was performed using a cellulose acetate membrane followed by mixing with colloidal silver nanoparticles for SERS detection, with the optimal ratio of protein to Ag NPs identified as 1:2. A multivariate statistical approach was used to analyze the SERS spectra, leading to the development of a diagnostic model. Clinical serum samples from 30 breast cancer patients and 45 healthy volunteers were analysed. The method showed an accuracy of 94.67 % in distinguishing the proteins of breast cancer patients from those of healthy individuals, demonstrating its effectiveness and reliability.

A microfluidic SERS chip (see chapter 2.1. for an introduction into microfluidic SERS approaches) has been developed for the detection of protein biomarkers [148]. The chip uses an aptamer-functionalized gold nanocrown array for specific target recognition and signal amplification. The method involves capturing protein markers with a specific aptamer and subsequently releasing a complementary aptamer strand bound with a Raman reporter molecule. The reduction in signal intensity correlates directly with the quantity of proteins detected. This study analysed clinical serum samples from 30 colorectal cancer patients and 30 healthy controls. The detection limits for hnRNP A1 and S100P were 0.031 pg/mL and 0.057 pg/mL, respectively, within 15 min. These findings demonstrate precision in identifying important protein biomarkers.

The study presents a detection method for protein biomarkers such as trypsin, pepsin, BSA, and haemoglobin, using Raman reporters and a novel glass capillary SERS probe with gold nanostars and polydopamine layers for selective recognition [36]. The sensor works in two steps: first, proteins are captured and then quantified based on the reduced access of a Raman reporter to the SERS substrate, resulting in a decrease in signal intensity. This technique allows the detection of Raman-inactive proteins, providing a simplified approach for point-of-care applications without the need for sample pre-treatment.

Despite its simplicity, direct detection of protein biomarkers faces limitations such as alterations in protein structure, Raman inactivity, and size constraints that limit access to SERS hotspots. Labeled methods, which offer greater specificity and sensitivity, dominate SERS-based protein detection. However, simplifying these processes remains a critical challenge.

4.3.3. SERS-based detection schemes in bacterial research

Direct SERS allows for direct fingerprinting of bacterial surfaces and their metabolites, providing detailed structural information and qualitative assessment. However, it may lack selectivity in complex environments. In contrast, label-based methods excel in identifying bacteria with greater selectivity and multiplexing ability.

Cofactors involved in the respiration of *Sulfurospirillum multivorans*, a microaerophilic organohalide-respiring bacterium, were detected [149]. The organisms were cultured with various electron acceptors, producing distinct SERS signatures for different cofactors. Cobalamin (vitamin B12) fingerprints were detected in bacteria cultivated with perchloroethylene (PCE) using silver substrates and membrane fractions, indicating active dehalogenation. This cofactor was not detected in cultures grown with fumarate, nitrate, or oxygen. Therefore, SERS can be used to identify specific metabolic activities. Moreover, a sandwich-based SERS platform is presented that uses a vancomycin-modified gold @ Prussian blue nanoparticle for bacterial detection in whole blood [150]. The platform includes 4MBN as an internal reference and 4MPBA as bacterial capture unit. The detection sensitivity is enhanced by focusing on the SERS signal of 4MBN in the biologically silent Raman region through large area mapping (Fig. 19). This method allows for the precise and selective detection of *Staphylococcus aureus* without signal interference.

Stable SERS nanotags using silver-coated hollow gold nanospheres (hollow Au@Ag) to detect cells and tissues were introduced [60]. The Raman reporter molecules are placed between the core and shell. The surface is coated with PEG to improve the biocompatibility. This enabled SERS imaging in two cell lines, Sk-Br3 and MDA-MB-231, facilitating the exploration of cellular dynamics. Subsequently, the technique was used to investigate the distribution of biomarkers, EpCAM, ErbB2, and CD44, using specific antibodies for targeted detection. This method supports cell viability testing and enables multiplex Raman imaging for identifying cancer cells, highlighting its potential in cell detection.

To conclude, in complex samples, separation of bacteria is necessary to eliminate interfering components and isolate bacterial cells. Techniques like membrane filtration are commonly employed. Additionally, recognition elements such as antibodies, aptamers, or molecularly

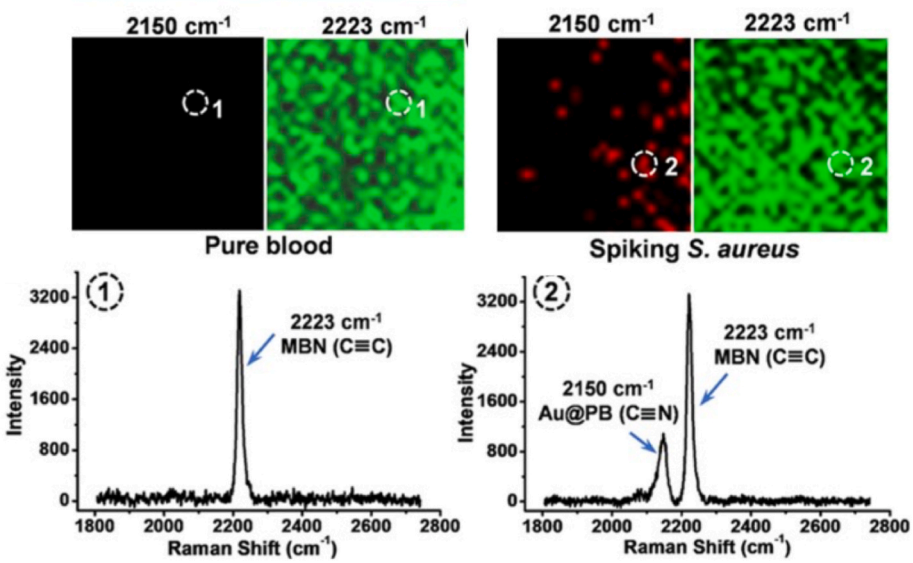

Fig. 19. SERS mapping of pure blood sample and after spiking with *S. aureus* for 2150 and 2223 cm^{-1} Raman modes, and their respective Raman spectra indicated at points 1 and 2 in the images. Reprinted with permission from [150]. Copyright 2021 American Chemical Society.

imprinted polymers are utilized to enhance the selectivity from similarly sized components.

4.3.4. Estimation of the pH value in cells

Accurate measurement of intracellular and extracellular pH is crucial for understanding cell composition and functions. Cellular pH changes affect metabolism, migration, and muscle tension, with different organelles exhibiting unique pH values. Monitoring cell pH is key to understanding disease mechanisms. The carboxyl, amino, and pyridine rings are effective SERS probes due to their responsiveness to pH variations and affinity for metal surfaces [151]. A novel 3D SERS imaging platform was developed to visualize changes in intracellular pH distribution in living cells. Gold nanostars functionalized with 4-MBA are used as stable SERS pH probes. These probes are coated with a cationic biocompatible polymer, poly-L-arginine hydrochloride, which enhances cell uptake and provides stability in the cellular environment due to its positive charge. Nanoparticles accumulated at perinuclear sites, and the associated local decrease in pH indicated diffusion from endosomal to lysosomal compartments. High-resolution 3D SERS imaging is used to visualize local changes in intracellular pH, such as acidification during nanoparticle endocytosis [152]. A further example presents SERS based pH sensing in cell cultures complemented by calorimetry [153]. The sensor is consisting of polyaniline-coated gold nanorods encapsulated within PEG diacrylate microgels. This dual-mode sensor can detect pH changes across a range of 1–12. The sensitivity of the method to pH is due to the protonation and deprotonation of polyaniline, which causes shifts in both the colour and Raman signal intensities of the microparticles. This approach allows for accurate pH monitoring by quantifying SERS in tumour cell cultures. The microgel encapsulation prevents contamination, ensuring measurement accuracy up to 0.5 pH units. This highlights the effectiveness of SERS in sensing cell pH values. Finally, a SERS-active glass nanopipette, designed with optimized geometry and a gold nanoparticle layer at the tip, enables precise intracellular analysis of single living cells [154]. Gold nanoparticles, functionalized with 4-MBA as a pH reporter, offer pH sensing from 6 to 8 with high sensitivity of 0.2 pH value units. This method tracks intracellular pH changes in HeLa cancer and fibroblast cells in response to external pH shifts. The HeLa cells were found to be more resistant to the external pH change, adapting to the less acidic environments. This plasmonic nanopipette represents a promising tool for single-cell analysis applications. In summary, the development of pH SERS probes requires a focus on signal stability and reliability. Additionally, constructing a protective matrix and improving biocompatibility are crucial for successfully implementing SERS-based pH sensing in cellular analyses.

4.3.5. Quorum sensing and microbial communication

The rapid detection of bacterial metabolites responsible for cell communication is important for the early diagnosis of bacterial infection and treatment with appropriate antibiotics. However, direct analysis of complex bacterial cultures using the conventional SERS approach is difficult. A paper-based 3D SERS substrate was designed establishing a facile one-pot Au electrodeposition protocol to directly detect pyocyanin, a signaling molecule of *Pseudomonas aeruginosa*, secreted in the culture medium, without pretreatment steps [155]. During one-pot Au electrodeposition in the presence of an Au precursor and culture medium, pyocyanin is captured in the interstitial voids of the 3D hydrogel matrix among growing Au grains, resulting in high amplification of the Raman signal intensity. Because the hydrogel skin excludes macromolecules like proteins in the culture medium, thereby maintaining an uncontaminated surface, they managed to achieve a direct and rapid detection of pyocyanin in a complex bacteria culture sample without any purification steps. Therefore, the pyocyanin concentration during bacterial culturing is easily and directly monitored, which is important for the early diagnosis of infection and in-depth studies of bacterial cell communication.

Similarly, Wang et al. applied SERS to monitor bioactive metabolites produced by ampicillin-resistant *P. aeruginosa* strains to identify the mechanisms underlying the antibiotic resistance [156]. The results indicated that the blue-green pigment pyocyanin dominates the metabolite signals and is significantly enhanced upon exposure to sub-minimal inhibitory concentrations of ampicillin (see Fig. 20). PYO accumulates during exponential growth and subsequently either diffuses into the culture medium or is consumed in response to nutrient deprivation. The SERS spectra further reveal that the production of some intermediate substances such as polysaccharides and amino acids is minimally impacted and that nutrient consumption remains consistent. Moreover, the intensity changes and peak shifts observed in the SERS spectra of non-PYO-producing ampicillin-susceptible *Escherichia coli* demonstrate that exogenously added PYO enhances *E. coli* tolerance to ampicillin to some extent. These results indicate that PYO mediates antibiotic resistance not only in the parent species but also in cocultured bacterial strains. The metabolic SERS signal provides new insight

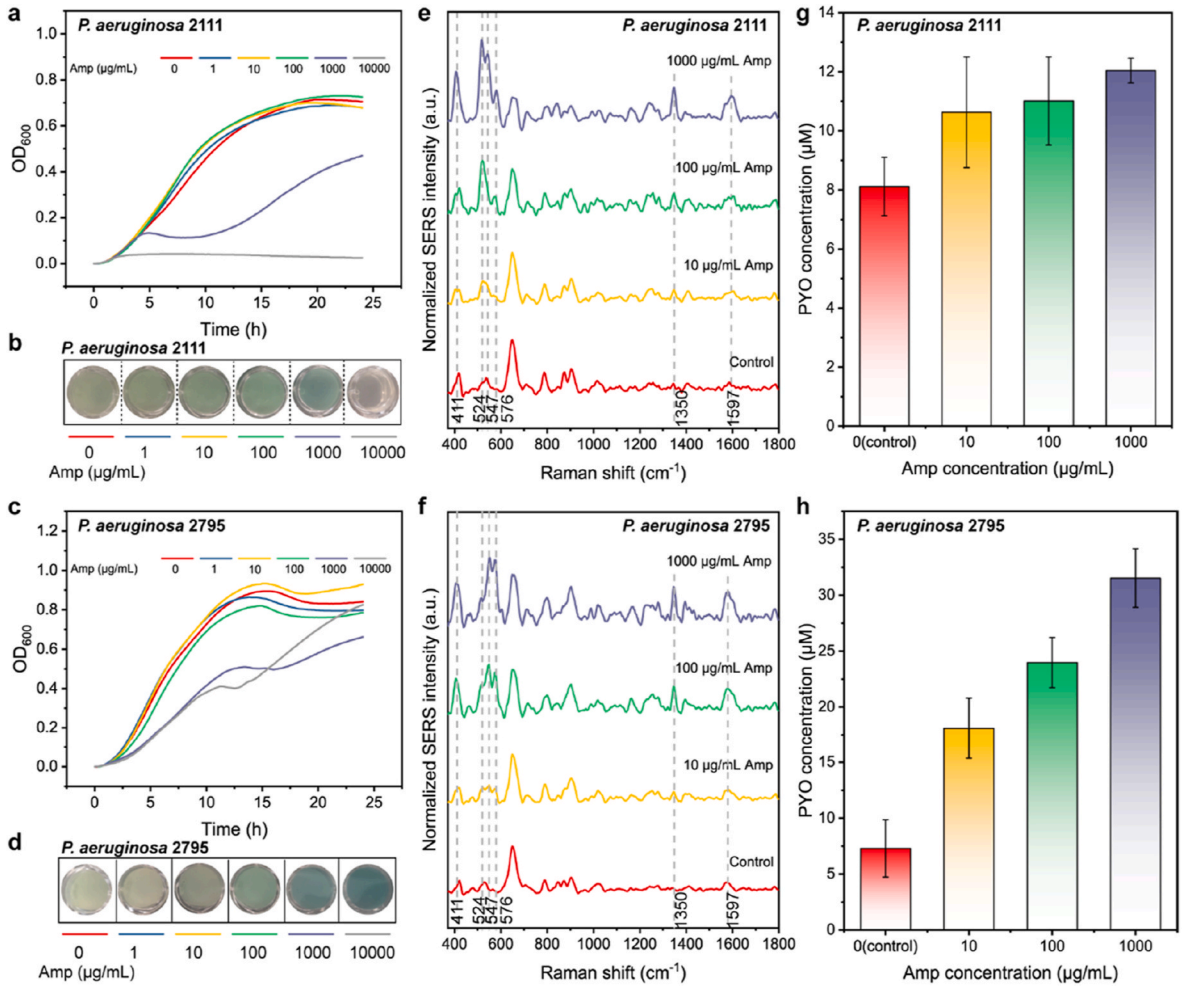

Fig. 20. Effect of ampicillin on *P. aeruginosa* strains 2111 and 2795 after 24 h. (a, c) Growth curves of *P. aeruginosa* 2111/2795 under varying ampicillin concentrations (0–10 000 μg/mL). (b, d) Plate color of *P. aeruginosa* 2111/2795. (e, f) SERS spectra of *P. aeruginosa* 2111/2795 metabolites after 24 h of cultivation. (g, h) Extracted pyocyanin concentrations in *P. aeruginosa* 2111/2795 plates under different ampicillin concentrations (0–1000 μg/mL). Reprinted with permission from [156]. Copyright 2023 American Chemical Society.

regarding antibiotic resistance with promising applications for both environmental monitoring and rapid clinical detection.

Biofilms are complex environments where matrix effects from components such as extracellular polymeric substances and proteins can strongly affect SERS performance. The interactions between SERS-enhancing Ag and Au particles and *ex situ* biofilms were studied, allowing systematic quantitative studies, where samples could be accurately diluted and analysed [157]. Strong signals from intrinsic marker compounds were found for the *Pseudomonas aeruginosa* and *Staphylococcus aureus* extracted *ex situ*, which the standard addition method showed were due to 2×10^{-3} mol dm^{-3} pyocyanin or the equivalent of 1×10^{-4} mol dm^{-3} adenine, respectively. The *ex situ* biofilms hindered aggregation of Ag colloids more than Au, but for both Au and Ag nanospheres the presence of the biofilm reduced SERS signals through a combination of poorer aggregation and blocking of surface sites. For Ag, the effect of lower aggregation was to reduce the signals by a factor of 2, while the site blocking gave a further 10 × reduction for adenine. Similar results were found for Au nanospheres with adenine, although these particles gave low enhancement with pyocyanin. Nanostars were found to be unaffected by reduced aggregation and also showed lower site blocking effects, giving more reproducible signals than those from aggregated particles, which compensated for their lower enhancement factor. These results provide a rational basis for selecting enhancing substrates for use in *in situ* studies, where the further complexity means that it is important to begin with well-understood and controllable enhancing media.

A feedback inhibition effect of high autoinducer levels on metabolite secretion in *Chromobacterium subtsugae* was detected using *in situ* spatiotemporal SERS profiling [158]. The hierarchical hydrophobic plasmonic array in agar medium is structured by oil/water/oil (OL/W/OH) triphasic interfacial self-assembly. The hydrophobic layer acts as a "door curtain" to selectively permit adsorption of a quorum sensing-regulated fat-soluble metabolite, i.e., violacein, and significantly blocks nonspecific adsorption of water-soluble proteins, etc. The SERS profiling proved that the diffusion of *N*-hexanoyl-L-homoserine lactone (C6-HSL) in agar medium quickly triggers the initial synthesis of violacein in *C. subtsugae* CV026 but surprisingly inhibits its synthesis in *C. subtsugae* ATCC31532. The latter negative response might be related to the VioS repressor of ATCC31532, which negatively controls violacein production without influencing the expression of the *Cvi*I/R quorum sensing system. Moreover, two sender-receiver systems are constructed by separately coculturing CV026 or ATCC31532 with *Hafnia alvei* H4 that secretes large amounts of C6-HSL. Expectedly, the cocultivation similarly triggers the initial synthesis of Vio in CV026 but seems to have a quite weak negative effect on the intrinsic synthesis in ATCC31532. In fact, the negative regulation in ATCC31532 might be affected by a diffusion-dependent concentration effect. The H4 growth and its secretion of C6-HSL are a slow and continuous process, thereby avoiding the gathering of local high concentrations. Overall, this study put forward an *in-situ* SERS strategy as an alternative to traditional

Table 1
Summary of SERS-based technological approaches in biosensing; their advantages, limitations and challenges as well as the assessment of possible commercialization.

SERS-based sensing system (technology)	Advantages	Limitations and challenges	Commercialization
SERS-based POCT: LFAs (indirect targeted SERS)	One-step, simple procedure which is widely applicable to different kinds of targets Requires low sample volumes Rated as consolidated, reliable technology	Requires recognition elements Pre-treatment occasionally necessary for complex matrices	Expected due to high technical readiness level
SERS-based POCT: Targeting biomarker, e.g. breath analysis (in/direct targeted SERS)	One-step, simple procedure which is widely applicable to different kinds of targets Volatile organic compounds are accessible	Requires molecular sensor modification	Expected due to well-established procedure and simple data readability
Microfluidic SERS systems	Handling of small sample volumes Fast measurements in high-throughput and high reproducibility Implementation of sample preparation, such as isolating and sorting and multiplexing due to well-engineered channel system	Requirement of (syringe) pump system might limit POC capabilities Appearance of memory effect in continuous flow analysis	Probable if portable and cost-effective microfluidic approaches are applied
EC-SERS-based systems	Potential-dependent SERS measurements for selective electrostatic enrichment of target analyte Miniaturization for small sample volumes Implementation of sample preparation steps	More complex software and instrumentation (e.g. potentiostat) required	Likely due to access to direct SERS information in complex biofluids
In vivo clinical SERS employing fiber-probes or SESORS approaches	Detection of biomarker, tumor margins, etc. *in vivo* Requires no sample preparation	Invasive technique requiring implementation of SERS substrate inside the human body	Challenging due to need for approval of implementation of SERS substrates

bioluminescent tools for a highly sensitive analysis of the spatiotemporal communication and cooperation in live microbial colonies.

Direct SERS has been proposed as a promising bacterial detection technique. However, the quality of the collected bacterial spectra can be affected by the time between sample acquisition and the SERS measurement. How storage stress stimuli influence the direct SERS spectra of *Pseudomonas syringae* samples stored in phosphate buffered saline was evaluated [159]. The results indicate that when faced with nutrient limitations and changes in osmatic pressure, samples at room temperature (25 °C) exhibit more significant spectral changes than those stored at cold temperature (4 °C). At higher temperatures, bacterial communities secrete extracellular biomolecules that induce programmed cell death and result in increases in the supernatant SERS signals. Surviving cells consume cellular components to support their metabolism, thus leading to measurable declines in cell SERS intensity. Two-dimensional correlation spectroscopy analysis suggested that cellular component signatures decline sequentially in the following order: proteins, nucleic acids, and lipids. Extracellular nucleic acids, proteins, and carbohydrates are secreted in turn. After subtracting the SERS changes resulting from storage, the authors evaluated bacterial response to viral infection. *P. syringae* SERS profile changes enable accurate bacteriophage Phi6 quantification over the range of 10^4–10^{10} PFU/mL. The results indicated that storage conditions impact bacterial label-free SERS signals and that such influences need to be accounted for and if possible avoided when detecting bacteria or evaluating bacterial response to stress stimuli.

5. Conclusion

In summary, SERS is a versatile and powerful tool in biosensing approaches. It is applied using main detection schemes defined as direct SERS, allowing molecules with high affinity to interact with the metallic nanostructured surface and thus contributing to the SERS fingerprint information, and indirect SERS, using SERS tags equipped with Raman reporter molecules and recognition elements for an increased specificity. Moreover, the SERS-based detection schemes can be distinguished via targeted and untargeted approaches, whereas targeted approaches using recognition elements such as antibodies, aptamers or imprinted polymers, whereas untargeted approaches are only selective based on the affinity of the molecules of interest towards the SERS sensing surface. As a rule of thumb, the larger the target (molecule) is, which is aimed to be detected via SERS, the more likely targeted approaches are applied by using specific SERS tags. Nowadays, a huge variety of SERS sensing platforms exists, e.g. microfluidics, strip assays or optical fibers, which are available at the point-of-care when combined with portable Raman read-out systems. In Table 1, we provide a summary of the technology platforms, their advantages and challenges as well as their potential in commercialization. Where it is not clearly stated, indirect targeted as well as direct targeted and direct untargeted SERS detection schemes are available. It should be noted that direct untargeted fingerprinting in SERS is despite its advantages in being a multi-marker approach and exploitation of all information available in the spectrum, its limitations and challenges arising due to limited numbers of biomolecules with high affinity towards the metallic surface contributing to the SERS signal, lack of specificity for the target molecule and inconsistent biological interpretation of results. We summarized various research fields, in which SERS-based biosensing is applied. This includes oncology, infectious diseases and other clinical relevant research fields such as cardiovascular diseases. However, SERS-based biosensing is not limited to medical or clinical research, it is also applied in environmental monitoring and assessment of the quality of food as well as biological orientated schemes such as identification of bacteria or quorum sensing. All of the discussed research topics have in common that the recorded SERS spectra require powerful artificial intelligence tools and machine learning for data analysis as the complexity of the SERS spectra is high in real matrices due to varying background composition or outnumbering concentration values of matrix components in comparison to the target molecules.

CRediT authorship contribution statement

Dana Cialla-May: Writing – review & editing, Writing – original draft, Funding acquisition, Conceptualization. **Alois Bonifacio:** Writing – review & editing, Writing – original draft, Conceptualization. **Alexey Markin:** Writing – review & editing, Writing – original draft, Conceptualization. **Natalia Markina:** Writing – review & editing, Writing – original draft. **Stefano Fornasaro:** Writing – review & editing, Writing – original draft. **Aradhana Dwivedi:** Writing – review & editing, Writing – original draft. **Tony Dib:** Writing – review & editing, Writing – original draft. **Edoardo Farnesi:** Writing – review & editing, Writing – original draft. **Chen Liu:** Writing – review & editing, Writing – original draft.

Arna Ghosh: Writing – review & editing, Writing – original draft. **Michael Schmitt:** Writing – review & editing, Funding acquisition. **Juergen Popp:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors have nothing to declare. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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