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Review

# A review of surface-enhanced Raman spectroscopy in pathological processes

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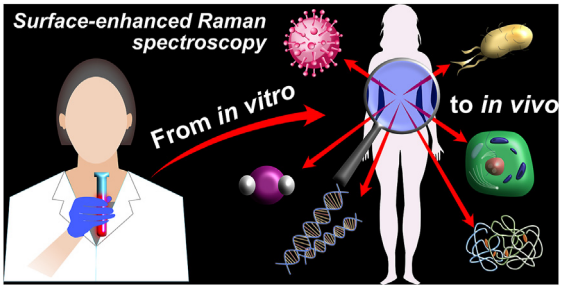
## HIGHLIGHTS

- SERS ultrasensitivity and specificity are critical to medical applications.
- We discuss details on different strategies to integrate SERS in pathology.
- Necrosis, apoptosis, and details behind cell-death can be accessed with SERS.
- Pathologies including inflammation, hypoxia, tumors, infections are thoroughly reviewed.

## GRAPHICAL ABSTRACT

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## ABSTRACT

With the continuous growth of the human population and new challenges in the quality of life, it is more important than ever to diagnose diseases and pathologies with high accuracy, sensitivity and in different scenarios from medical implants to the operation room. Although conventional methods of diagnosis revolutionized healthcare, alternative analytical methods are making their way out of academic labs into clinics. In this regard, surface-enhanced Raman spectroscopy (SERS) developed immensely with its capability to achieve single-molecule sensitivity and high-specificity in the last two decades, and now it is well on its way to join the arsenal of physicians. This review discusses how SERS is becoming an essential tool for the clinical investigation of pathologies including inflammation, infections, necrosis/apoptosis, hypoxia, and tumors. We critically discuss the strategies reported so far in nanoparticle assembly, functionalization, non-metallic substrates, colloidal solutions and how these techniques improve SERS characteristics during pathology diagnoses like sensitivity, selectivity, and detection limit. Moreover, it is crucial to introduce the most recent developments and future perspectives of SERS as a biomedical analytical method. We finally discuss the challenges that remain as bottlenecks for a routine SERS implementation in the medical room from *in vitro* to *in vivo* applications. The review showcases the adaptability and versatility of SERS to resolve pathological processes by covering various experimental and analytical methods and the specific spectral features and analysis results achieved by these methods.


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**Contents**



## 1. Introduction

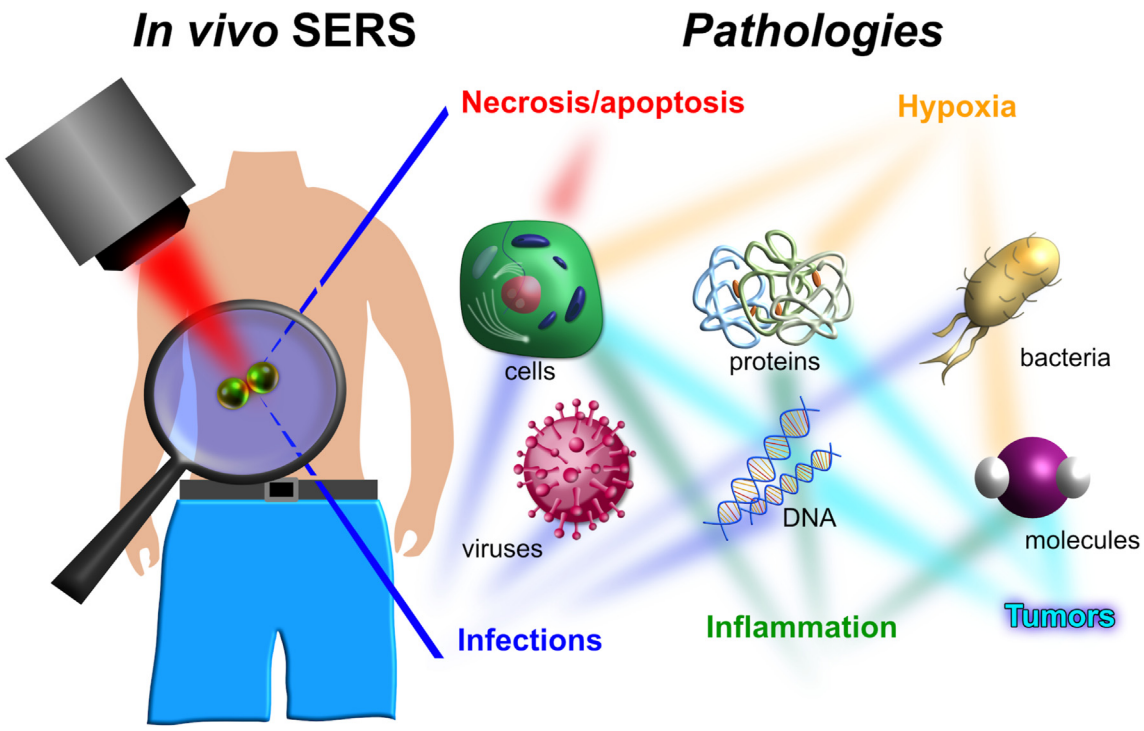
Analytical methods are an integral part of modern medicine, and first and foremost, in diagnosing pathological processes. The typical processes in Scheme 1 diagnosed with laboratory methods include hypoxia, inflammation, infections, necrosis, apoptosis, cancer. The therapy's success depends on the correct and fast diagnosis of the pathology, and its assessment now almost always requires confirmation by laboratory or instrumental methods. Recently, SERS emerged as a promising approach for real-time diagnosis [1]. SERS was established as a highly sensitive method, but its major advantages include the capability of performing both *in vitro* and *in vivo* real-time analyses and the simultaneous use of nanoparticles (NPs) as therapeutic agents. This review aims to introduce developments in *in vivo* and *in vitro* SERS-based approaches with good potential for the investigation of pathological processes illustrated in Scheme 1. Namely, we performed a comparison of conventional and emerging SERS diagnosis methods (Table 1). First, we discuss the basics of SERS, the main types of substrates and nanoparticles used for SERS measurements for characterizing living systems. Then, pros and cons are revealed and discussed. In the main section, after reviewing recent developments, we conclude with our take on *in vivo* and *in vitro* applications' perspectives. Finally, we discuss SERS's potential in clinical practice beyond controlled laboratory studies, how far modern medicine is from using SERS methods to identify pathological processes in patients, and our opinion on the most promising proposed directions.

Nowadays, different approaches for analyzing proteins and other pathology markers exist (see Scheme 1). We list them in Table 1. The limit of detection of classic laboratory methods is poor compared with SERS, except liquid chromatography-mass spectroscopy (LC-MS/MS), but its high response time and the impossibility of the use *in vivo* could promote Raman spectroscopy in-clinic diagnostics.

Liquid chromatography (LC) combines separation and mass analysis of components in a liquid medium. The components are distributed between two immiscible phases. LC methods are used alongside different instruments, including mass-spectrometry. Modern analytical systems have high LOD (down to pg/mL) and specificity that open the door to detection and quantitative analysis of endogenous compounds (hormones, vitamins, lipids), drugs, medicines, and their metabolites, volatile organic compounds, *etc.* in biological tissue [8].

Mass-spectrometry (MS) works with induced ion currents that make it possible to distinguish components based on mass to charge ratio, calculate their concentrations, and chemical identification of samples. MS has limited clinical diagnostics use, mostly for toxicology research (drugs [19] and doping control [20]) and infection diagnosis [21]. Nevertheless, the most popular applications lie in drug production, experimental genomics, and proteomics [22]. However, this method is challenging to handle, has high costs, requires highly skilled specialists, and has limited throughput.

Immunochemical methods are a set of techniques aimed at target protein identification based on antigen-antibody-specific reactions. This approach is typically coupled with markers that are used for detection. The detection approach can be classified as radioimmunoassay, immunoenzyme, immunoluminescent, or immunofluorescent based on the markers used. The high sensitivity and specificity of immunochemical methods (ELISA, Western blot, flow cytometry, immunohistochemistry, *etc.*) allow us to employ them in clinical laboratories for diagnosis. The most spread




**Scheme 1.** Different pathologies and biological analytes *in vivo* SERS detects and identifies.

applications are detecting serum proteins, hormones, infection, inflammation, tumor markers, and some medicinal substances [23].

Electrophoresis is based on the electric field separation of substances with different mobility when exposed to external electric fields. It separates molecules of various sizes, spatial configurations, electric charges, and secondary structures. In practice, electrophoresis is used to investigate body liquids such as urine, serum, neurolymph, pleural, pericardial, lacrimal fluid, and quantitative analysis of isoenzymes [20,24,25].

UV–vis is an optical method based on wavelength-dependent absorption and emission of light. Absorption spectra obtained during the experiment are characteristic for each substance. The application of UV–vis in clinical medicine is measurements of the concentration of proteins, nucleic acids, medicinal substances, and medium pH.

**Table 1**
Laboratory analysis methods comparison.

| Method | Acquired information | Limit of detection (LOD) | Typical analysis time | Potential of the use *in vivo* |
|---|---|---|---|---|
| Raman | Vibrational spectra of molecule | μg/mL [2] | From minutes [3] to seconds [4] | ✓ |
| SERS | Vibrational spectra of molecules, but the signals are enhanced by the presence of SERS-active substances (Au, Ag, *etc.*) | Mostly from ng/mL to fg/mL [5] | Less than 1h [5] | ✓ |
| LC-MS/MS | Sample composition based on mass to charge ratio [6] | ng/mL - μg/mL [7] | From minutes to seconds [7] | ✗ |
| | Detection and analysis of medicines, hormones, drugs, metabolites, lipids, organics *etc.* [8] | down to pg/mL [8] | down to 3h [8] | ✗ |
| Enzyme-linked immunosorbent assay (ELISA) | Determine the concentration of hormones, peptides, and proteins [9]. Identify the acceleration, specificity of the reaction [10]. | ng/mL [11] | 30–60min [9] | ✗ |
| Electrophoresis | Detection of proteins, medicines. Molecular composition, cancer diagnosis. | μg/mL [12] | 90-60min [12] | ✗ |
| Ultraviolet–visible spectroscopy (UV–vis) | Concentration measurements of proteins, nucleic acids, different medicines, and pH control [13] | μg/mL [14] | 5min [14] | ✓ |
| Nuclear magnetic resonance (NMR) | Molecular composition of samples | mg/mL [15] | 5min [16] | ✗ |
| Polymerase chain reaction (PCR) | Specific DNA or RNA sequences | copies/μL [17] | 1–3h [18] | ✗ |

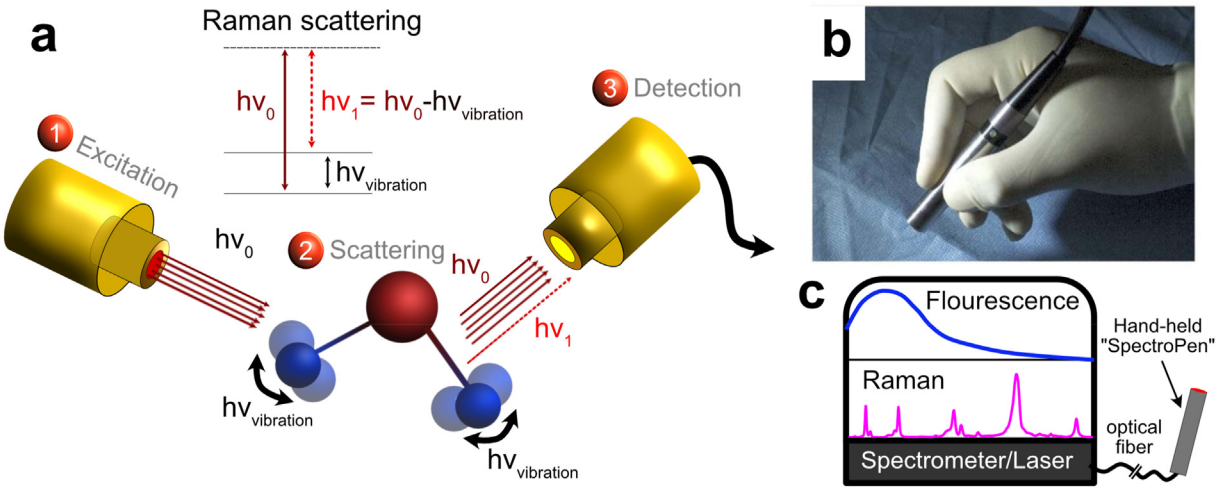



**Fig. 1.** (a) Visualization of Raman scattering process, (b) SpectroPen in operator's hand during the surgery, (c) Schematic diagram of SpectroPen. Adapted with permission from Ref. [26]. Copyright (2010) American Chemical Society.

## 2. Surface-enhanced Raman spectroscopy basics

SERS stands for surface-enhanced Raman spectroscopy. It is based on the inelastic (Raman) scattering of light. As illustrated in Fig. 1, the frequency of scattered photons changes upon interaction with matter in this process. Laser irradiation with frequency $\upsilon_0$ (Fig. 1a) interacts with outer-shell electrons of molecules producing inelastically scattered photons with a different frequency $\upsilon_1$. The difference between the initial and the resulting frequencies equals a vibrational frequency of the molecule $\upsilon_{vibration}$ under study. The spectra can be used to analyze biological samples with the identification of different molecular components. This identification is possible because the molecule provides a unique spectral response called “chemical fingerprint” in Raman spectroscopy. Each spectral band corresponds to a particular vibrational mode's energy, including C—C or N—N bands that might not be visible with infrared spectroscopy (IR) spectroscopy. Moreover, compared to IR spectroscopy, Raman spectra are not affected by water content and can be realized as a portable solution for diagnosis and intraoperative monitoring. Raman spectroscopy versatility is evidenced in applications like Spectropen (Fig. 1b) and others [4,26], making it possible to identify the composition of substances in a simple manner (Fig. 1c).

However, conventional Raman has very low sensitivity due to the low probability of the inelastic scattering process. For example, the light elastically scattered from a piece of paper is 10 times more intense than inelastically scattered photons. SERS is the most efficient approach for increasing the Raman signal intensity reaching the ultimate sensitivity level of single-molecule detection [27]. This spectroscopic implementation involves using various metallic nanostructures that provide Raman signal enhancement thanks to plasmonic resonances' excitation. Plasmons are coherent oscillations of the electron gas. In the absence of external excitation, the electron gas is uniformly distributed over the NP's volume (Fig. 2a). Their excitation with light makes the electron gas shift from its steady-state (Fig. 2b) and drives plasmonic oscillations. Significant local enhancement of the electromagnetic field is achieved by matching the electromagnetic field frequency and plasmon's fundamental frequency. As a result, the Raman signal can increase up to $10^6$ times [28]. This mechanism is called electromagnetic enhancement [29]. Another amplification contribution to SERS is the chemical enhancement mechanism [30,31] attributed to a change of molecular polarizability due to the appearance of new optical transitions induced by molecule-metal hybridization. This type of enhancement often involves charge transfer between molecule and metal, resulting in the formation of a metal-molecule complex, like covalent bonding or chemisorption. In addition to these effects, the relative metal-molecule orientation also influences the Raman spectra in SERS which could ultimately be exploited as a local probe for biomolecule-metal interactions [32].

SERS has good potential in biomedicine because of its high sensitivity and specificity. The SERS enhancement factor *in vivo* is up to $10^7$ [33], allowing the detection of substances at concentrations down to $10^{-15}$ M [34]. The literature shows multiple SERS applications in biomedicine, including glucose sensor [35,36], gout and pseudogout diagnostics [37], DNA sensor [38], tumor detection [39], malaria diagnostics [40], and so on. The analysis of samples in liquid mixtures and express-analysis are essential benefits of this method.

## 3. SERS methods applied to pathological processes

One of the essential points for SERS implementation is to place the detected molecules directly in a "hotspot" (Fig. 2c). Hotspots are the regions of space with the strongest amplification of electromagnetic fields. For this purpose, the SERS substrates with deposited noble metal nanostructures or colloidal NPs are used. The reason behind the use of noble metals is that their plasmon resonance is in the visible and near IR spectral range, while the resonant frequency for most other metals lies in the UV range. SERS substrates are films with plasmonically active nanostructures, while colloidal solutions comprise NPs dispersed in a buffer solution. For SERS measurements using nanostructures, samples must be located on the SERS active surface or (biocompatible) substrate that can be implanted directly into the tissue under study. In this case, the electromagnetic field source should be an IR laser due to the tissue transparency window for wavelengths from 650 nm and above. Another *in vivo* implementation of SERS is injecting colloidal solutions with plasmonic NPs directly into an organ or tissue with the Raman spectra acquired using an IR laser. The fabrication of plasmonic particles with resonance in the IR region was improved during the last 15 years by introducing new designs of SERS active structures. One of them is nanorods with two resonances, unlike the spherical particles, one weak in the visible range and the second strong in the IR [41]. The first is related to electron oscillations along the short nanorod axis, while the second is along the longitudinal.

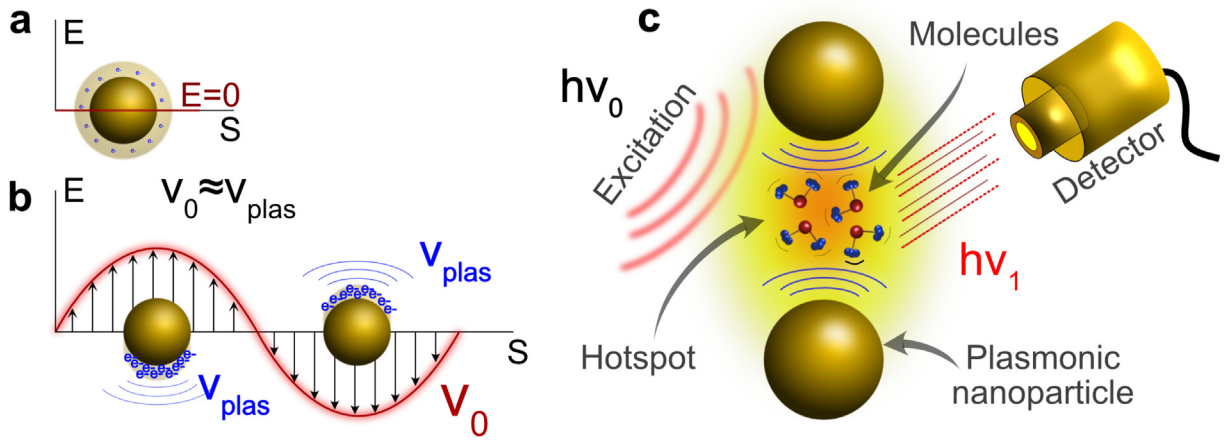



**Fig. 2.** Illustration of (a) electrons in a nanoparticle without illumination (E = 0) and (b) plasmonic effects with illumination (E(t)). (c) molecules in a hotspot and their ultrasensitive detection with SERS.

The benefit of this material is the easy-tune resonance that could be adjusted by changing the length to width ratio [42]. Applying other materials such as aluminum and copper to adjust the plasmon resonance [43] is in principle possible if the amount of metals is below the toxicity threshold. Another emerging approach is changing the optical properties of the substrate by creating a gradient photonic crystal to adjust the optical response [44]. This chapter reviews the state-of-the-art most promising analytical SERS-based strategies reported in the literature focusing on pathological processes.

### 3.1. Substrates with nanostructures (including in vivo applications)

For biomedical applications, SERS substrates should provide a high enhancement factor, homogeneity, and reproducibility. There are tons of options reported to fulfill these requirements [45,46]. A classical technique film over nanospheres (FON) is based on self-assembled nanosphere layers used as a template for metal deposition (Fig. 3a). This method provides higher enhancement than previous solutions due to the triangles' spiky corners that result in strong electric field enhancement [46]. Van Duyne [47] and Bartlett [48] contributed a lot to developing this approach to describe the fabrication process and characterize the substrates with appropriate methods like SEM and Raman spectroscopy.

The protocol involves electrodeposition of gold through a template of polystyrene latex spheres assembled on the surface. After the electrodeposition, the polymer spheres are removed by dissolving in dimethylformamide (DMF). The SEM images of the resulting substrates demonstrate the dependence of cell size on the diameter of polystyrene spheres and high homogeneity (Fig. 3b). Elongated plasmon structures with bidirectional plasmon resonances can be formed by a range of techniques, including self-organization on pre-patterned substrates [49]. SERS implementation *in vivo* is attractive since it has opportunities for real-time

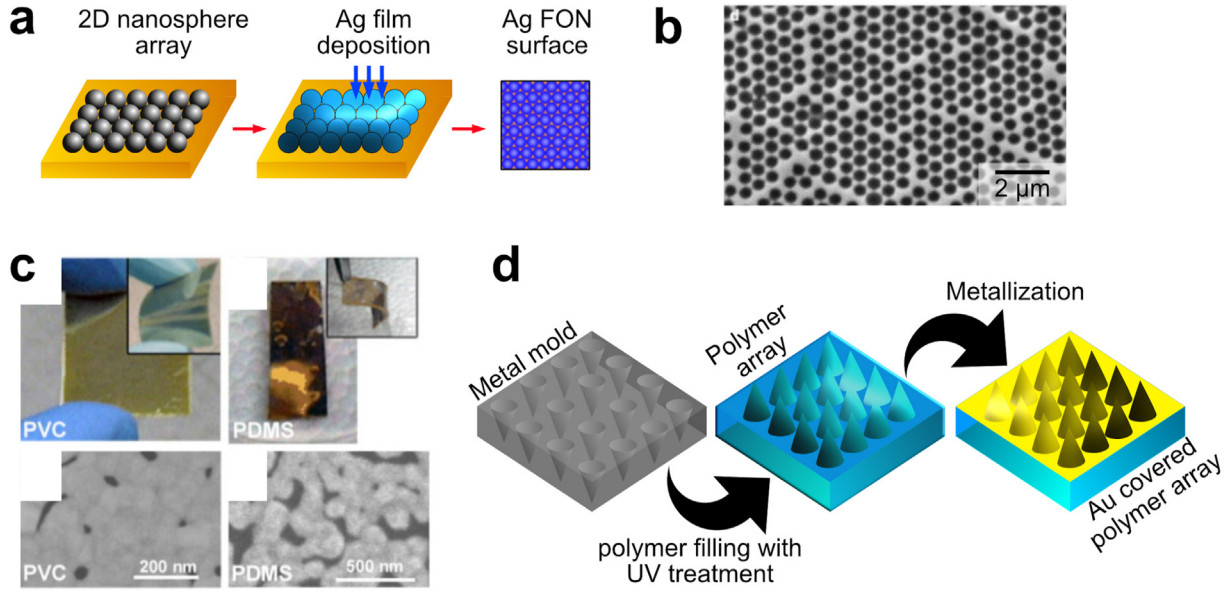



**Fig. 3.** (a) Illustration of nanosphere lithography with self-assembled monolayer formation. Adapted with permission from Refs. [35,36] Copyright (2006) American Chemical Society, (b) SERS substrate, fabricated by electrodeposition of gold on nanosphere template [54], (c) photographs of Au/Au films on different polymers [33], (d) roll-to-roll UV nanoimprint lithography process illustration [55]. Adapted with Attribution 4.0 International (CC BY 4.0) license. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

monitoring and simplification of clinical trial procedures. However, SERS-active platforms based on traditionally used materials can lead to local or full-body toxicity or provoke an immune response. Therefore the development of biocompatible substrates, functionalized with SERS-active NPs based on non-metallic structures like indium-tin-oxide (ITO) [50,51], flexible polymers [33,52], and hydrogels, is an important step forward for *in vivo* applications [53]. Furthermore, integrating plasmonic nanostructures in polymer substrates is critical to make SERS substrates that conform to the body shape, as shown with gold nanostructures on PVC and PDMS in Fig. 3c. Polymer-based SERS substrates fabrication technology like imprinted polymers and roll-to-roll UV nanoimprint lithography became the equivalent of FON for obtaining solid SERS substrates. Nanoscale stamps for polymer structuring are at the core of imprinted polymers technology. On the other hand, roll-to-roll UV nanoimprint lithography uses casting on a predefined structure with the polymer filling the casting mold followed by further consolidation and NP metallization (Fig. 3d).

Some SERS applications require an increase of specificity and selectivity. For these purposes, one could functionalize substrates with antibodies or aptamers [56] (Fig. 4a). For instance, decanethiol/mercaptohexanol functionalization [35] was used for selective linking with antigens, target molecules, or glucose. This functionalization increases sensitivity by selective adsorption on metal NPs. Also, there are multiplex sensors with detection capability for different substances. These multiplexed sensors are possible thanks to a complex sensor functionalization described by Rodriguez-Lorenzo *et al.* (Fig. 4b) [57] and reviewed by Zeng *et al.* [58]. Besides the sensor functionalization with aptamers, the other exciting part of this strategy is the use of lateral flow assays described in a recent review by Kim *et al.* [59].

### 3.2. Nanoparticles

The crucial component of SERS is the nanostructure used for Raman signal enhancement. The most used materials are plasmonic nanoparticles, for which the enhancement depends on the nanoparticles' size, shape, and spatial arrangement. Some strategies used pre-patterned surfaces to deposit plasmonic nanostructures with the possibility of controlling the plasmon resonance, density, and directionality in linear assemblies; see for example [49,61–63]. Other approaches combine plasmonic nanoparticles with photonic crystals to provide an additional boost to the Raman signal while achieving a broadband resonance response in a single substrate, as shown by Dedelaite *et al.* [44]. However, even without being plasmonically active, the substrate itself affects the system's optical properties, resulting in different cell components becoming selectively enhanced [64]. The combination of plasmonic nanoparticles with other enhancement systems like carbon nitride functionalized with polymer brushes [52] is gaining momentum due to the possibilities to create multifunctional SERS platforms, including flexible dual SERS and electrochemical sensors [65]. The other approach to applying SERS in biomedical systems is the use of colloidal NPs (Fig. 5). The method efficiency also depends

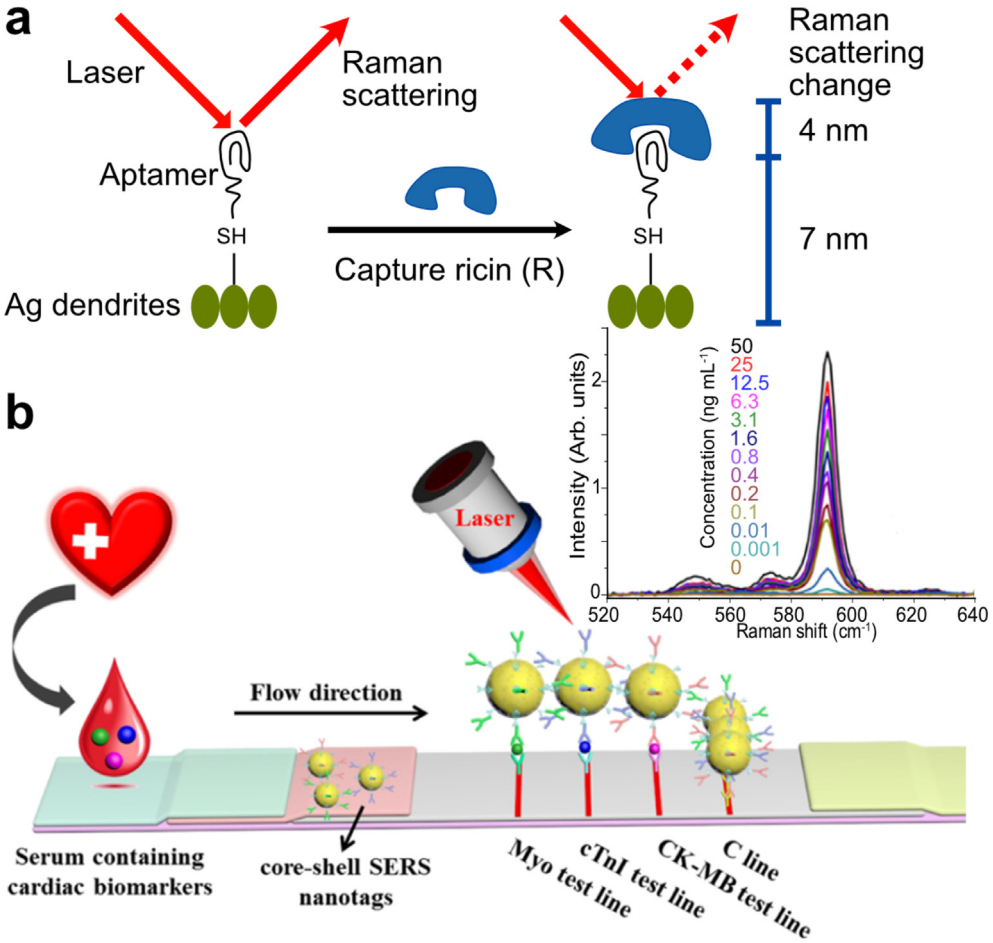



**Fig. 4.** (a) Illustration of aptamer-functionalized substrate use in Raman analysis. Adapted from Ref. [56], (b) design of multiplex sensor applications. Adapted from Ref. [60].

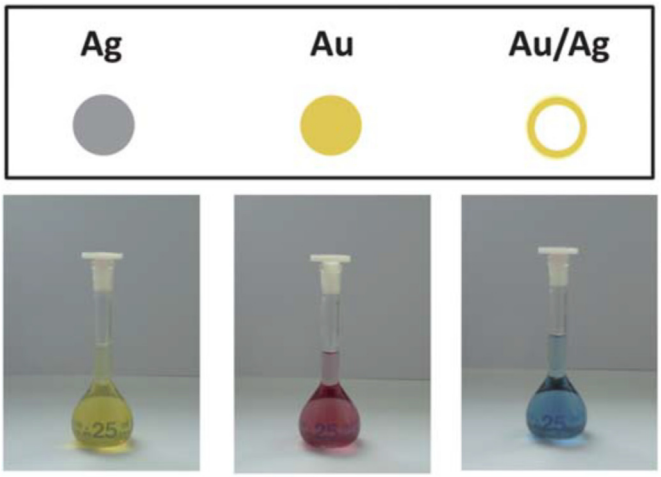



**Fig. 5.** Variety of gold and silver colloids for *in vivo* SERS applications. Adapted from Ref. [74]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

on the material, shape [66], size of the particle [67], and colloid dispersity [68]. As mentioned above, colloidal particles could be delivered directly to tissues or organs for various purposes for biomedical applications. However, these colloids' biocompatibility is still debated because of body immune response and NP accumulation in organs [69].

The effectiveness of colloid gold with different shapes was widely discussed by Klebtsov *et al.* [70]. The key point of using these structures is creating sharp edges on the metallic core and gaps between them to create, tune, and enhance the hotspots. In this work, they describe the growth of the gold layer (islands) on the thiolated aromatic molecules and Raman-active polymers (spacers) attached to the plasmonic active cores of different shapes (spherical, rods, and stars). This approach consists of three stages: design of the core, functionalization with polymer or aromatic molecules, and creating the metal shell. For example, the maximum enhancement factor achieved by Gandra's group is $1.7 \times 10^{11}$ [70,71]. They used Au core with a diameter of 20 nm, 1,4-benzenedithiol spacer of 0.62 nm with further Au coating.

Another strategy that is on the leading edge of hot research topics is the use of core-shell structures. These nanostructures are extensively used for SERS due to localized surface plasmon resonance properties [72], with the inner core and outer shell made of different materials. Core-shell particles could be divided into two types: with ultrathin and thick shells [72]. The particles with thin shells mainly “borrow” the electromagnetic field of the active core to enhance the Raman signal of the analyte, while thick-shell particles are usually functionalized to expand SERS applications. Other classifications consider the shape, material, electrical properties, magnetic properties, and others [73]. The SERS enhancement of core-shell nanoparticles depends on the core size and the shell thickness. These features with different materials that could be used for core or shell (not only metals) allow producing nanoparticles that ideally target a particular application.

Performing *in vivo* measurements is quite a complicated task due to three key factors: 1) complex chemical composition of biologic tissue, 2) weak Raman signal of some important analytes, and 3) body immune response. One of the prospective ways to minimize the impact of those issues is using SERS labels or reporters. This method is based on bonding dyes, fluorophores [75], or other SERS active molecules with the analyte of interest to detect it further. The SERS detection occurs by tracking the strong Raman active label attached to the target molecule. Colloids (including SERS-labels) could be functionalized with biocompatible polymers such as polyethylene glycol [39] (as shown in Fig. 6) or other biocompatible materials to obtain high specificity, high sensitivity, and reduce immune response [60,76].

An alternative method to increase the sensitivity of colloids is aggregating NPs. As a result, the molecules get trapped in the most intensive hotspots in between NPs. Furthermore, the agglomerate formation allows manipulating plasmon resonance peak position by varying aggregate size [77]. At present, there are two implementations of this method: the use of magnetic nanoparticles (MNPs) or coagulation.

MNPs are 1–100 nm sized nanoobjects that can be manipulated and concentrated by an external magnetic field. Often MNPs are combined with plasmonic nanoparticles to form magnetic plasmonic particles [78]. MNPs like $Fe_2O_3$ dipped in a colloid of SERS active NPs can provide enhancement factors of about $10^6$-$10^7$ [79]; as we will see in this review, the combination with MNPs offers one of the most powerful approaches to improve the limit of detection for *in vivo* and *in vitro* SERS.

Different compounds such as sodium citrate could be implemented to induce aggregation [77]. Among these, the so-called Frens' method is widely applied, based on aggregate synthesis by citrate reduction [77]. This method employs the synthesis of colloidal NPs ranging from 21 to 146 nm in diameter, thus artificially changing the width of the plasmonic band by random NP aggregation. The key research problem is whether the broadening of the plasmonic band suppresses the strong plasmonic amplification by the aggregate formation and the effect of NP size tuning. Therefore, the use of aggregation methods poses some implementation difficulties. On the other hand, the advantage is that by varying the reagents ratio, NPs, and their concentrations, one can adjust the aggregates' size or shape [80]. Therefore, besides good selectivity and the ability to drastically enhance sensitivity, the SERS approach is convenient for *in vivo* analysis and is open for various modifications, making it flexible for a broad spectrum of applications.

## 4. Necrosis and apoptosis

There are two main metabolic processes that result in cell death, necrosis and apoptosis. Here, we briefly discuss these processes and some examples of the information that SERS provides and a critical discussion of the benefits and limitations.

Necrosis is unplanned cell death caused by strong damaging factors - physical, chemical, biological, *etc*. Different mechanisms lead to the destruction of cell membranes and cytoplasm release in the intracellular medium. Inflammatory mediators and chemoattractants are released upon the cells' death, attracting leukocytes to the necrotic focus. White blood cells support the inflammation that usually accompanies the necrosis process.

Contrary to necrosis, apoptosis is a programmed, genetically controlled process of cell death. During apoptosis, the cell divides itself into a few apoptotic bodies, each containing a fraction of the original cell's cytoplasm and DNA, which is then absorbed by the surrounding cells. The major differences between necrosis and apoptosis are an intact cell membrane and the absence of inflammation. Apoptosis is a normal physiological process; however, it can be evidence of a pathology, for example, a viral infection [81].

These differences between the two cell death processes provide a unique spectral fingerprint in Raman spectroscopy. Before jumping to SERS, getting information about the two different processes and their differences with Raman microscopy is critical. This was done using different kinds of cells and methods to induce cell death [82–85]. These investigations showed that necrosis is

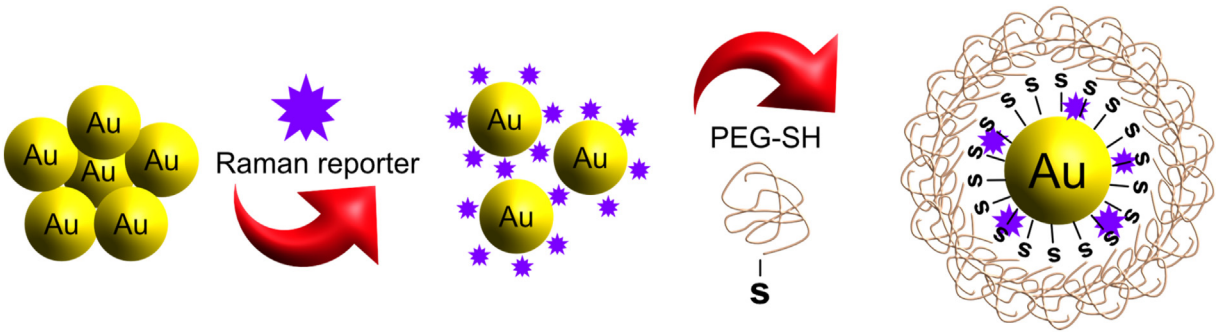



**Fig. 6.** Design of pegylated Au NPs for *in vivo* tumor targeting and spectroscopic detection. Redrawn based on [39].

characterized by an increase in intensities of protein signals [82,83], while for apoptosis protein signals can be decreased [86] or not significantly differ from normal cells [84]. An increase in cytochrome *c* [85] and phospholipids signals [84] was also observed in apoptotic cells. The late apoptosis stage is also accompanied by a decrease in the cell's DNA content [82,84].

In addition to the chemical specificity of Raman microscopy, SERS has the advantage of amplifying the signal from molecules located near the SERS-active NPs. This characteristic is exploited to investigate cell death based on detection of ligands for cellular death receptors (such as tumor necrosis factor alpha, or TNF-α) or death-inducing signaling complex (DISC) components (RIP1, FADD, pro-caspases), which assemble on activated death receptor [87]. However, most of the experiments focused on detecting TNF-α [88–90]. For example, the combination of Ag NPs and magnetic beads coupled with antibodies for TNF-α [88] form sandwich-like structures in the presence of the ligand. Then the concentration was increased by an external magnetic field right before Raman spectroscopy was performed. This method's LOD appeared to be 1 pg/mL, which is ten times better than other SERS-based methods (~9.69 pg/mL) or the corresponding ELISA analysis (10 pg/mL).

According to Zhang *et al.* [91], apoptosis can be identified by following the signals from Bcl-2 group proteins or phosphatidylserine, which moves to the cell's surface at the early stages of apoptosis. Later, this was used by Zhou *et al.* [92] to detect the early stages of apoptosis *in vitro*. Their method was based on immersing cells in an $AgNO_3$ solution and then adding a reducing agent ($NH_2OH \cdot HCl$). The positively charged $Ag^+$ ions self-assembled into NPs on the surface of apoptotic cells due to negatively charged phosphatidylserine. This method provides a distinct Raman signal from the cells that are undergoing apoptosis (Fig. 7a). One potential limitation of this method for *in vivo* conditions is that other negatively charged species such as proteins or other phospholipids could also reduce silver ions. In that case, the SERS spectra are no longer representative of apoptosis.

Apart from colloidal NPs, substrates with plasmonic nanostructures can be used instead [93]. Apoptosis, induced by the addition of Triton X, was evaluated from the O–P–O groups' signal intensity characteristic of DNA. In apoptotic cells, the drug's Raman intensity reduced slowly and almost faded away on the third day of the experiment. Alternatively, it is possible to place SERS-active NPs in a specific place inside the cell to amplify as many signals from cell components as possible [94–96]. For example, Au NPs conjugated with arginine-glycine-aspartic acid (RGD), and nuclear localization signal (NLS) protein activated the relocation of NPs in the perinuclear space in the HSC-3 cell during apoptosis induced by adding $H_2O_2$, see Fig. 7b [95]. The location of the NPs near the nucleus led to the collection of essential signal related to cell condition - such as the DNA Raman signal [96]. The same approach allowed researchers to describe spectral changes during necrosis and apoptosis in HSC-3 and MCF-7 cells [97]. Peaks from the phenylalanine ring increased significantly during apoptosis due to tertiary or quaternary protein structure denaturation that led to the release of phenylalanine. Necrotic cells showed an intensity increase of most substances, even from DNA, because they were released from the nucleus and appeared in the cytosol near the nanoparticles. The distinctive characteristic of living cells is the strong signal from disulfide bands located at 490-510 $cm^{-1}$ (Fig. 7c).

The SERS investigation of caspase family enzymes, which activate on the final steps of apoptosis from inactive procaspases, breaking down a variety of proteins and leading the cell to its death, offers another venue to evaluate specific cell death processes [98–100]. Au nanorods functionalized with a marker molecule with protein-ligand for Caspase 3 reacted upon apoptosis, resulting in decreasing the Raman marker intensity. The LOD of this method was reported to be 1.99 ng/mL [99]. A reversed strategy is also possible [100] by using Au NPs functionalized with molecules of two different proteins that exposed negatively and positively charged amino acids after protein-ligand breakage by interaction with Caspase 3. This caused the NPs to aggregate into large clusters, leading to significant enhancement of Raman signal, and improving the LOD up to 1.5 ng/mL.

Thus, Raman spectroscopy allows implementing different strategies to detect and monitor cell death. For newcomers to the field wanting to identify with SERS whether cells undergo apoptosis or necrosis a first check is to look for the Raman peaks from phospholipids or cytochrome C that represent apoptosis, or look for enhanced peaks from proteins that would point to cell death by necrosis. The decrease of DNA peaks was attributed to late stages of apoptosis due to its cleavage by nucleases into short fragments which later will be packed in apoptotic bodies. We should notice that with SERS, the spectra can vastly differ from each other depending on the nanotag location. On the other hand, this feature allows us to collect signals from specific cell structures or molecules, offering great flexibility to study cellular processes including programmed cell death.

Advance detection of necrotic processes in the internal organs is of the utmost diagnostic and clinical significance. The spectral changes are expressed enough to be detected by classical Raman spectroscopy while SERS offers better sensitivity as well as higher localization. The main signs of necrosis *in vivo* are a pronounced inflammatory reaction, fever, and the appearance of serological markers. Also, postmortem examination often involves tissue microscopy to help determine the cause of death. At the same time, the study of apoptotic processes is in demand almost exclusively in experiments to study the initial signs of damage and is much harder to detect. In this regard, SERS's use might make recording the earliest stages of injury possible since the method in its various

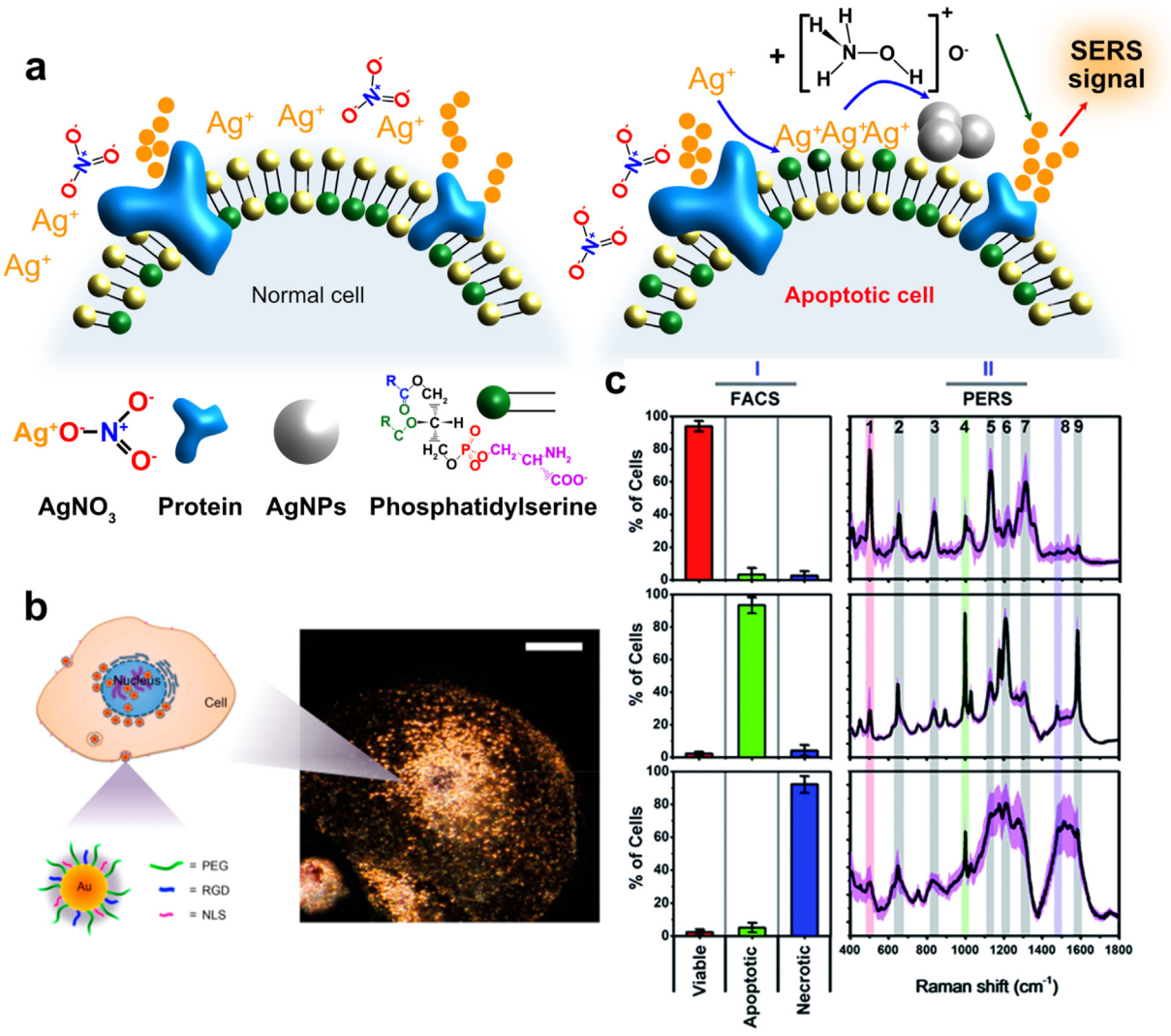



**Fig. 7.** (a) Self-assembly of Ag NPs on the surface of the cells during the early stages of apoptosis. Reproduced with permission from Ref. [92]. Copyright 2016, RSC Publishing. (b) Endocytosis of NPs by cancer cells and their visualization inside them by dark-field Rayleigh imaging. Adapted with permission from Ref. [95]. Copyright 2014, ACS Publications. (c) Schematic representation of collecting Raman signals from the absorbed NPs and peak determination to each type of cell death. Adapted with permission from Ref. [97]. Copyright 2017, RSC Publishing.

versions has comparable or even higher sensitivity and operation speed. SERS application to study cell death processes *in vivo* is not there yet. The limiting factors are the high cost of equipment and high personnel specialization; but this situation also offers fertile grounds and opportunities for new research.

## 5. Inflammation

Inflammation is a complex body response to injury followed by clinical and biochemical changes. Clinical signs of local inflammation are redness, swelling, local temperature rise, pain, and tissue dysfunction, which could be diagnosed during a medical examination. Non-specific symptoms include headache, fever, and decreased performance. The body biochemistry is also affected by increased inflammation markers concentration in blood, such as acute phase proteins (C-reactive protein (CRP), procalcitonin, fibrinogen, haptoglobin, *etc.*) and leukocytosis that are monitored to establish the therapy efficiency. Infection, cancer, and autoimmune diseases are often accompanied by inflammation making identification of specific inflammation biomarkers a very attractive and powerful approach. In this section we discuss how SERS can be used to detect systemic signs of inflammation *in vitro* and potentially *in vivo*.

### 5.1. Analysis of the inflammation markers in situ

When tissue gets damaged, during the first stage of the inflammation process called the alteration phase, the primary inflammation mediators emerge from destroyed cells, immune cells, and blood. These mediators trigger acute inflammation and thus can be used as targets to study inflammation *in situ* with SERS.

The approaches for detecting early markers of inflammation *in vivo* with SERS, such as histamine [101,102] and bradykinin [103] are in their initial stages of development. There is almost no research on the evaluation of those substance levels during different pathological processes. In most cases, only the presence of the target analyte is determined. A few works considered the possibility of serotonin determination with SERS, though not as an inflammatory mediator but as a neurotransmitter [104,105]. Moody *et al.* [105] introduced a model approach to study the spatial

distribution of neurotransmitters in the brain with SERS and spatially offset Raman spectroscopy (SORS). The spectroscopy was performed on the model brain of a cat directly through the animal's skull using Au NPs functionalized with melatonin, serotonin, and adrenaline. The characteristic peaks of each neurotransmitter were non-invasively detected in all spectra in 50 mM and 100 μM concentrations, which is sensitive enough to detect serotonin rise in serum during pathological processes [106]. During the second phase of inflammation (exudation and emigration), different immune cells that are the source of inflammatory mediators (*i.e.,* cytokines, chemokines, chemoattractants, *etc.*) migrate to the lesion site. These cells can also be targeted for SERS detection.

Bronchial asthma is an excellent model for cytokine expression by leukocytes. To assess inflammatory changes in bronchus in children with such disorder [107] SERS allowed quantifying the concentrations of 37 cytokines with multiplex analyzer MAGPIX (BIORAD, USA) in saliva samples from healthy and ill children with the signs of bronchial obstructions, and a correlation between interleukin (IL)-8, IL-10, and sCD163 concentrations was found. Among all biomarkers, the characteristic peak at 1326 $cm^{-1}$ was present only for IL-8, and the signal's intensity was significantly higher in children with bronchial asthma than in the healthy group. The asthma group's spectra also showed increased peak intensities in the regions related to cytochrome *c*, amide III, lipid, and triglyceride hydrocarbon chains (1050–1127 $cm^{-1}$). Thus, one of the cytokines was validated in saliva with SERS. The following multivariate principal component analysis (PCA) allowed them to estimate the predictive values of this non-invasive diagnostic method (sensitivity was 85%, specificity 82%, and accuracy 84%). Such implementation of SERS in natural saliva in a simple and non-invasive way can provide crucial information about the endotype of bronchial inflammation and help diagnose and monitor asthma which, with no known cure or even a gold standard for diagnosis, affects hundreds of millions of people resulting in about half a million deaths per year [108].

Macrophages' primary task is to remove dead cells' particles and pathogenic microflora from the inflammation site. They also play a central role in realizing innate immune system response and initiation of adaptive response. The destruction of pathogenic microorganisms and the representation of their antigens to the lymphoid cells requires acidic media of the phagolysosome. Insufficient pH levels lead to prolonged progression of bacterial infections, such as tuberculosis, pseudotuberculosis, *H. pylori*-associated gastritis, *etc*. [109–111]. In this regard, a SERS approach to evaluate the acid production by macrophages' phagolysosomes was developed [112] by monitoring the Raman spectra of Au nanoparticles functionalized with para-mercaptobenzoic acid (p-MBA). This approach is simple to implement and shows high sensitivity due to the pH-sensitive Raman probe integrated on the nanoparticles.

An elegant experiment was carried out by Dugandžić *et al.* [113] who created Au nanoprobes that were actively absorbed by macrophages due to mannose molecules on the surface. As a result, they observed the migration of macrophages to the sites of inflammation where the SERS signal intensity was the highest, the atherosclerotic plaques. Based on these results, the authors suggested differentiating stable and unstable plaques (the latter often leads to thrombosis), which have high prognostic significance and make this approach valuable for potential *in vivo* applications. Several researchers [114–118] investigated atherosclerotic plaques in different developmental stages *ex vivo* using Raman spectroscopy rather than SERS. For example, Liu *et al.* [114] could distinguish fibrolipid, calcified and unstable atherosclerotic plaques, obtained from dead patients. Rygula *et al.* [118] detected changes in phenylalanine and tyrosine concentration ratio in atherosclerotic endothelium extracted from apolipoprotein E/low density lipoprotein receptor (ApoE/LDLR)-deficient mice.

During atherosclerosis pathogenesis accompanying inflammation, low-density lipoproteins and very-low-density lipoproteins (LDL and VLDL) accumulate inside the vascular wall. SERS performed *in vivo* on different arteries of rabbits on a cholesterol diet confirmed this lipoprotein accumulation *in vivo*, see Fig. 8a and b [119]. This example also illustrates the benefits of extending SERS beyond the conventional Raman system, since to obtain spectra inside the rabbit's arteries it was necessary to design a catheter waveguide system for laser excitation and Raman signal collection.

As mentioned above, an essential element of the immune response during atherosclerosis pathogenesis is the migration of leukocytes in tissues, which is closely related to the vessel's wall condition. Cell adhesion molecule expression on the endothelium becomes enhanced during endothelial activation, and the dysfunction that follows. Those molecules include the intercellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1 (VCAM-1) that assure strong leukocyte adhesion to the endotheliocytes. McQueenie *et al.* [120] detected ICAM-1 on the endothelial cells *in vitro*, *ex vivo*, and *in vivo* with SERS and compared this approach to flow cytometry (FC), immunofluorescence, and two-photon fluorescence microscopy. Specific detection of ICAM-1 was achieved by conjugation of Au NPs, covered by a Raman reporter molecule, a $SiO_2$ layer, and ICAM-1 antibodies. In every experiment, SERS showed a better signal-to-noise ratio than conventional methods. A similar experiment was carried out by Noonan *et al.* [121], but, aside from ICAM-1, two additional molecules, biomarkers VCAM-1 and P-selectin, were selected for simultaneous detection by SERS. An important advantage of SERS is the narrow and characteristic peaks for different molecules and reporters, which allow implementing a multiplex approach in a single experiment. The evaluation of multiple molecules was achieved by associating each antibody/NP with a unique Raman reporter. Several antibody/reporter pairs were used simultaneously: anti-ICAM-1-BPE (1,2-bis(4-pyridyl) ethylene), with a characteristic peak at 1202 $cm^{-1}$; anti-VCAM-1-PYOT (5-(pyridine-4-yl)-1,3,4-oxadiazole-2-thiol) (1575 $cm^{-1}$); anti-P-selectin-PPY (4-(1H-pyrazol-4-yl)pyridine) (952 $cm^{-1}$) (Fig. 8c). Endotheliocytes of the human coronary artery, which were stimulated with 10 ng/mL TNF-α for 24 h and immobilized with acetone, showed multiple detection of selected molecules with SERS, which was also confirmed by immunofluorescence microscopy. In the *ex vivo* experiment, fresh segments of coronary arteries were used from patients' hearts who received a heart transplant. Different sections of these segments (with atherosclerotic plaques and without) were spectroscopically studied. Immunofluorescence from ICAM-1 and P-selectin reporters was observed in sclerotic and healthy regions, VCAM-1 only in sclerotic tissue. Multiplex SERS-spectroscopy showed the same result but with increased intensity for ICAM-1 and P-selectin in the sclerotic region. *In vivo* non-invasive multiplex SERS-spectroscopy and SERS-microscopy was realized on the site of human fat tissue transplanted to mice. SERS results with NPs injected intravenously showed the expression of the desired molecules in the mice vessel's endothelium.

The so-called “respiratory burst” - one of the many weapons of the immune system against pathogens - is a process when macrophages produce a large number of free radicals such as hydrogen peroxide, superoxide anion, hydroxyl ion, *etc*. [122]. Being extremely chemically active compounds, free radicals bind to the bacteria cell wall, interacting with lipids and membrane proteins, the cytoplasm of damaged or infected cells, disrupting cell function and ultimately causing cell death. Cui *et al.* proposed a new SERS approach to the study of ROS [123]. The development of a new nanoprobe based on gold/para-aminothiophenol/hemin made it

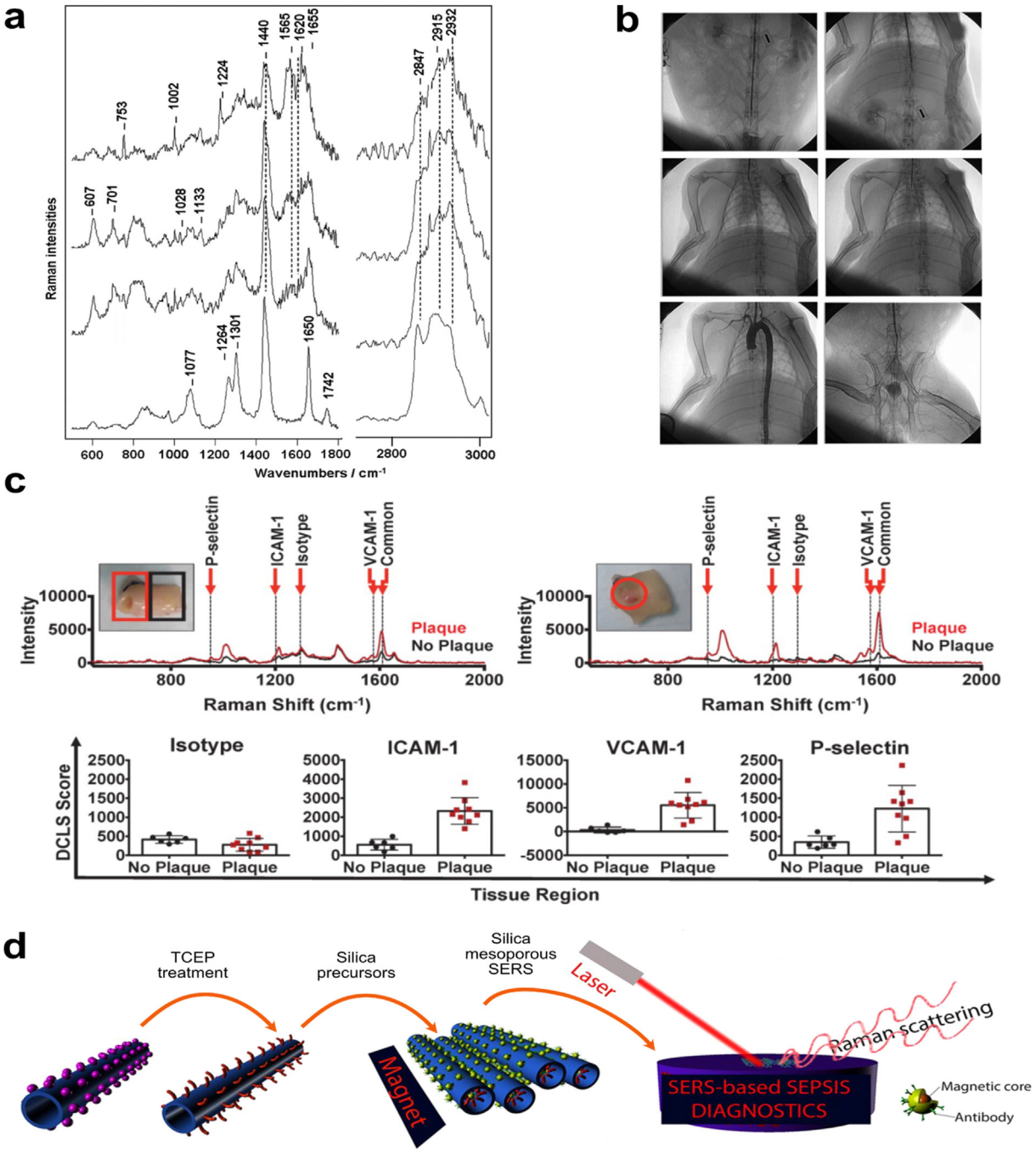



**Fig. 8.** (a) Raman spectra of arteries of a rabbit on a cholesterol diet. The lower spectrum shows an increased amount of lipids inside the artery's wall, suggesting an atherosclerotic process. Reproduced with permission from Ref. [119]. Copyright 2012, ASC Publications. (b) Characteristic peaks for each of vascular inflammation biomarkers and comparison of their intensities in spectra of healthy coronary artery samples and with atherosclerotic plaques. Reproduced with permission from Ref. [119]. Copyright 2018, Theranostics. (c) X-ray images of *in vivo* Raman spectroscopy of arteries in a rabbit on a cholesterol diet. Reproduced with permission from Ref. [121]. Copyright 2012, ASC Publications. (d) Assemble of SERS-active magnetic mesoporous structures for multiple detections of septic markers. Reproduced with permission from Ref. [129]. Copyright 2016, Elsevier.

possible to detect $H_2O_2$, $O_2^{\bullet -}$, $^{\bullet}OH$, $ROO^{\bullet}$, and $^1O_2$ simultaneously. The study of two *in vivo* models showed high efficiency of monitoring ROS levels during tumor development and allergic dermatitis progression.

Once again, SERS approaches based on functionalized nanoparticles allow non-invasive multiplexing *in vivo* research of the stages of the inflammation process and its important elements, such as macrophage movement, making the real-time monitoring of disease progression much more straightforward. It also appears quite helpful to precisely localize the site of inflammation that accompanies most of the pathological processes, including socially significant atherosclerosis, thus helping the surgeon locate and remove the plaque. However, while being much more sensitive than Raman spectroscopy, SERS requires applying nanoparticles in the patient's body, which nowadays needs further research - there is still a concern about the toxicity of NP made from noble metals.

### 5.2. Systemic inflammation

Inflammation is a local pathological process. However, when the immunological reaction is too strong, cytokines get released in the bloodstream. This is when inflammation becomes systemic. Thus, it becomes possible to monitor the patient's condition by testing her blood samples. The first stage of the inflammation process can have a vastly different duration - from a few seconds to several days, depending on the damaging agent. However, already on the first day, the concentration of biochemical inflammation markers changes, giving the first opportunity for monitoring a disease course. Among the various infectious and inflammation markers, the most frequently used is CRP. Conventional methods for CRP concentration measurements in plasma or serum are radial immunodiffusion, immunoturbidimetry, and nephelometry. SERS selective detection of CRP at extremely low concentrations (down to $10^{-17}$ M) [124] was possible through the use of atomic flat gold nanosurface with adsorbed CRP antibodies. After adding the CRP solution, the gold surface was modified with Au NPs conjugated with CRP antibodies creating hotspots that made such an extremely high sensitive detection possible.

SERS quantitative CRP detection in blood plasma was used as an early indication of radiation-induced inflammation in rhesus-monkey [125]. Animals were split into three groups, one as a control group, and the other two were irradiated with sublethal (4 Gy) and lethal (8 Gy) radiation doses. CRP concentration levels were monitored during 80 h with immunochromatographic analysis. For this analytical approach, nanoparticle markers were composed of Au/Ag core-shell NPs with built-in hotspots and embedded Raman reporters. CRP levels reached their peak values after 12–24 h of the experiment. These experiments proved the possibility of SERS-based modified immunochromatographic analysis application as fast and precise biodosimetric analysis for sorting patients affected by radiation. Besides CRP, future SERS studies could consider other relevant biomarkers of radiation damage, including IL-6, serum amyloid A protein, and amylase [126].

Inflammation is a key indicator of sepsis, which is one of the most dangerous systemic inflammatory reactions that without timely treatment leads to organ failure and death. Sepsis is caused by the generalization of local infectious processes or by disruption of the organism's regulation response to infection. Therefore, prompt and reliable sepsis diagnosis is a crucial and challenging task. There are several sepsis biomarkers, CRP discussed above, procalcitonin (PCT), presepsin, IL-6, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), soluble urokinase plasminogen activator receptor (suPAR), and CD64. CRP and procalcitonin are actively applied in clinical practice, while the rest of the molecules are being investigated for sepsis diagnosis and prognosis [127,128]. Several researchers investigated the SERS detection of these sepsis-specific markers. For this, SERS substrates were developed based on the M13 phage [129]. Its cysteine groups were reduced to active thiol groups, and then modified to form mesoporous templates the structures with pores 2–50 nm in diameter – ready to be anchored with gold magnetic nanostars (Au-MNS) *via* thiol-gold interactions creating magnetic mesoporous structures. These structures were used as a substrate for detection of three septic markers in serum samples: CRP, PCT, and sTREM-1 by the SERS tags conjugated to antibodies for each marker, detection limits of 27 pM, 103 pM, and 78 pM respectively. This SERS approach has a strong potential for the clinical diagnosis of sepsis. (Fig. 8d).

Another set of proteins associated with inflammation was detected with a vertical flow assay based on nanoporous anodic aluminum oxide (AAO) functionalized with antibodies to CRP, IL-6, SAA (serum amyloid A), and PCT [130]. After adding the test sample, SERS-nanotags (Au/Ag/polyethylene glycol (PEG)/Raman dye antibody) were applied to this AAO matrix. This approach allowed the simultaneous SERS detection of CRP, IL-6, SAA, and PCT with impressive LODs of 53.4, 4.72, 48.3, and 7.53 fg $mL^{-1}$, respectively.

The utmost representation of blood biomarkers of inflammation belongs to CPR. Other substances, such as IL-1β, IL-6, IL-8, TNFα, *etc.*, are also used for inflammation diagnostics, but not as broad as CPR [131]. SERS can change the balance of biomarkers or find new ones.

Here, SERS provides a measurement of different inflammatory mediators and markers *in vivo*, *in vitro*, and *in situ*, in concentrations far lower than LOD offered by conventional methods. Furthermore, it is possible to observe leukocytes and their interaction with the inflammation site components or find atherosclerotic plaques *in vivo.*

Inflammation is a protective and adaptive reaction that occurs with a variety of tissue damage closely related to many other pathological processes. Often, based on the severity of the inflammatory reaction, a conclusion can be drawn about the degree of organ damage. Acute inflammation occurs with extensive tissue trauma, which has a characteristic clinical picture and is accompanied by a high concentration of inflammatory markers in the plasma that are easily determined by conventional methods. However, in chronic inflammatory reactions, the high sensitivity of SERS could be crucial since the level of inflammation markers is low, and thus, it is often not suitable for monitoring inflammatory processes with conventional methods.

An additional advantage of SERS is its ability to determine chronic inflammation not only by the level of plasma markers (such highly sensitive tests are already available, for example, CRP) but also *in situ* in a specific target organ during a biopsy. This would be invaluable in diagnosing and treating autoimmune diseases, several slow chronic inflammatory diseases, glomerulonephritis, allergic reactions, diabetes mellitus, *etc*. Those conditions are also of high importance for modern healthcare, and, of course, there are works related to diagnostics by Raman spectroscopy. However, due to length considerations, it is not possible to describe every experiment in this review. Some additional attention should be paid to detecting early inflammation markers to predict the beginning of the inflammatory site emergence.

## 6. Hypoxia

Hypoxia is defined as the disruption of tissue oxygenation [132]. This pathological process can accompany many diseases and pathophysiological conditions such as stroke, atherosclerosis, cancer, or inflammation [133]. There are two standard methods of hypoxia detection to date. Pulse oximetry is a non-invasive method that allows collecting information about light absorbance (λ of incident light is 660 and 940 nm) by oxygenated hemoglobin, which depends on oxygen blood saturation. Another way is the direct measurement of gases in arterial blood when the level of oxygen partial pressure in blood is below 60 mm Hg. By following a range of intracellular biochemical processes and, as a result, the presence of specific biomarkers during hypoxia, we can investigate this pathological process and its scale. For example, during the immunohistochemical analysis, the HIF1α transcription factor is evaluated because its level increases in hypoxic cells during different metabolic disruptions [134].

Another well-studied hypoxia marker is the changes in the activity of some intracellular reductases [135–137]. At low pH conditions accompanying local tissue hypoxia, the increase in nitroreductase activity changed the SERS spectra from Au nanorods functionalized with 4-nitrothiophenol (4NTP) [136]. In normal conditions, the $NO_2$ group provides a strong Raman signal, but in the activated enzyme's presence, this group gets reduced to $NH_2$, thus decreasing the 4NTP signal. This strategy was tested with lung

cancer samples in different oxygen concentration solutions. Instead of 4NTP, Qin *et al.* [137] used the azobenzene derivatives reduction during hepatocytes ischemia. It leads to the detachment of molecules from the nanoprobe resulting in signal drop. Its intensity compared to the signal from carbon serves as an internal standard (Fig. 9a).

Another technique is direct detection of pH or redox potential change in hypoxic cells [138–141]. For example, Nguyen *et al.* [142] put a gold nanolayer with 4-nitrothiophenol molecules, which are sensitive to redox potential change on the nanopipette surface. This probe was used *in vivo* to study hypoxia in tumors in rats. Moreover, it appears that the level of redox potential differs significantly in benign and malignant tumors, which makes not only detection but also identifying different forms of cancer possible (Fig. 9b).

Numerous research groups recently highlighted the enzymatic properties of Au NPs, see the review by Liang *et al.* [143]. Hu *et al.* [144] applied those "nanozymes" features combined with their ability to enhance Raman signals to determine glucose and lactate levels. To accomplish that, they put glucose (or lactate) oxidase and Au NPs in the metal-organic framework MIL-101. Moving through its highly porous wall, the substrate gets oxidized and releases $H_2O_2$. This molecule leads to the activation of SERS-inactive leucomalachite green to the SERS-active malachite green with Au NP acting as an artificial enzyme for this reaction. The more glucose/lactate is present in the medium, the more hydrogen peroxide gets released, and the more signaling molecules become activated

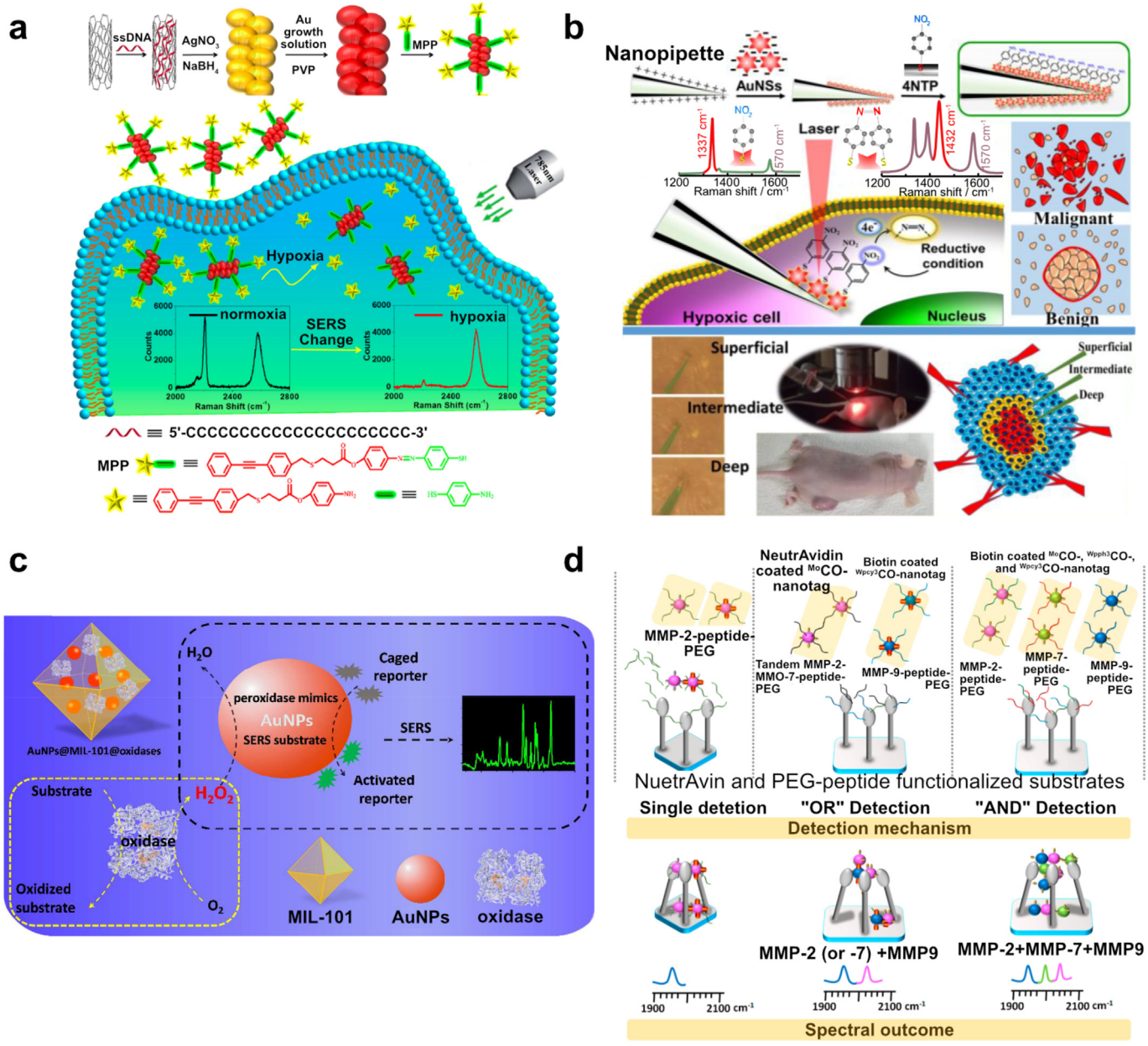



**Fig. 9.** (a) Structure and principle of work of Ag–Au carbon nanoprobe for detection of hepatocyte ischemia. Adapted with permission from Ref. [137]. Copyright 2019, ASC Publications. (b) Detection of hypoxia with nanopipette, covered in a nanolayer of gold. Adapted with permission from Ref. [142]. Copyright 2019, Wiley. (c) Function of SERS-active "nanozymes" for detection of hypoxia. Adapted with permission from Ref. [144]. Copyright 2017, ASC Publications. (d) *In vitro* detection of matrix metalloproteinases by cleaving corresponding protein substrates on carbon nanopillars and Au NPs. Adapted with permission from Ref. [157]. Copyright 2017, ASC Publications. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

enhancing the Raman signal from malachite green. This method allowed tracking the glucose and lactate levels in a rat's brain *in vivo* during ischemia and reperfusion and with a preliminary injection of the antioxidative drug astaxanthin and without it, all while quantifying the exact level of those molecules in tumors (Fig. 9c).

Schlabritz-Loutsevitch *et al.* [145] used Raman spectroscopy to analyze the signal from fetal perfusate modified to model placental hypoxia. For that, the orbital raster scanning technique was used, where the laser moves during analysis, scanning a predetermined sample's region. By comparing spectra from normoxic and hypoxic fetal perfusate collected at different times after retrieval from the patient, it was shown that the hypoxia could be evaluated with the most accuracy in 1 h after collection.

Hypoxia is the primary pathological process in the development of various cardiovascular diseases (CVD). Despite a decline in worldwide mortality from those disorders, it remains the major cause of death worldwide, with 9 million fatalities in 2016 [146]. Electrocardiography (ECG) is the gold standard for CVD diagnosis; though it only approximately localizes ischemia (on which heart wall is the ischemic site and how deep it is), it does not visualize the exact extension of the disease [147].

Besides, ischemia also occurs during cardioplegia [148], where ECG is not useful because of the absence of myocardium contraction during operation. Considering this, SERS could be developed to monitor the heart condition during an open-heart surgery, which significantly impacts cardiac surgery. For example, Brazhe *et al.* [149] observed ischemia in a perfused rat heart by registering intense Raman signals from reduced cytochrome *c* and deoxymyoglobin and decreased oxymyoglobin signal intensity. Similar results were obtained on cardiomyocytes *ex vivo* in normo-, hypo-, and hyperoxic media [150]. Aside from that, it is possible to diagnose ischemic events by detecting specific biomarkers such as troponin I and myoglobin. There are examples related to the detection of these substances *in vitro* with various substrates [60,139,151]. Chen *et al.* [139] performed spectroscopy on a patient's saliva to track acute myocardial infarction. After centrifugation, the sample was placed on an aluminum substrate and analyzed with Raman spectroscopy. Although the spectra from healthy and ill persons are similar, a slight but consistent difference between the positive and negative analytes becomes visible with a sensitivity of 80.0% and specificity of 80.6%.

Stroke-related mortality takes second place in worldwide morbidity, with almost 6 million deaths in 2016 [146]. Therefore, searching for specific stroke biomarkers in blood serum appears to be a promising option for diagnostics. However, this search is extremely challenging due to insufficient specificity and sensitivity of modern diagnosis methods, the hematoenchephalitic barrier that prevents the movement of biomarkers from the stroke area to blood, and heterogeneity of the disease [152]. Combining conventional ELISA and SERS to determine the levels of biomarkers, such as S100-β protein [153,154] and neuron-specific enolase (NSE) [155] is a promising way to solve those limitations of conventional methods.

Furthermore, Raman spectroscopy's analytical capabilities can be improved using biomarker panels, that is, a complex of biomarkers used to distinguish a specific illness. A particular example of this approach showed sandwich-like structures on a paper substrate that made the qualitative analysis of NSE and S100-β possible [156]. Each biomarker had a set of specific antibodies associated with either the substrate or Au@Ag NPs with Nile blue A and 4-mercaptobenzoic acid (4-MBA) connected to NSE and S100-β antibodies, respectively. In case of a positive result, the biomarker gets captured by the antibodies on the paper's surface, and then the free antibodies with NPs and corresponding dye attach to them, making hotspots with a strong Raman signal. LOD is 0.05 ng/mL$^{-1}$ for S100-b and 0.01 ng/mL for NSE. SERS studies evidenced that matrix metalloproteinases (MMP) concentration is slightly higher in patients' serum due to stroke [157]. SERS substrates were made from nanopillars and various NPs, based on gold, tungsten, and molybdenum carbonyls. The sensing mechanism is based on carbonyl's interaction with different nanoparticles that induce shifts of Raman peaks for each NP type and hotspot formation that enhances the signal for specific MMP. This method was successfully demonstrated *in vivo* analysis of blood plasma from rats 1 h after a stroke was induced with a LOD of 0.05 ng/mL, 0.05 ng/mL, and 0.1 ng/mL for MMP-2, MMP-7, and MMP-9, respectively with better overall performance than ELISA, electrochemical, and fluorescence methods (Fig. 9d).

Thus, we reviewed how the high sensitivity of SERS makes it possible to successfully determine hypoxia markers both *in vitro* and *in situ*, opening the door to more effectively monitoring postoperative and other severe conditions, such as cancer, stroke and heart attack. The main advantage of SERS in contrast to the analysis of the general markers of hypoxia in blood is the ability to pinpoint the affected area, potentially - during surgery or other clinical procedures. Nevertheless, using SERS, the noninvasive identification of hypoxic focus related to any pathological process still represents a tricky but demanding task that is crucial for accurately diagnosing and prescribing the respective treatment.

However, Raman spectroscopy appears to be quite sensitive for detecting oxy- and deoxygenated hemoglobin [158], making it a potential substitute for some conventional methods for blood oxygenation monitoring, especially in the brain [159]. Commercial Raman devices for blood monitoring have been in the field of medicine for quite a long time, but they are limited in measurements (only relative measurements of oxygenation can be seen) and fairly inaccurate [159,160]. A new generation of Raman devices has emerged recently, capable of precise, non-invasive measurement of broad cerebral blood characteristics, including absolute cerebral oxygen saturation. Unfortunately, those instruments require further clinical testing and are not yet implemented in practice [160,161].

## 7. Tumors

A tumor is a pathological process manifested by uncontrollable cell replication, usually accompanied by decreased differentiation. Malignant tumors are the 2nd mortality cause worldwide [162], with substantial socioeconomic implications.

Histological examination of the primary tumor site by biopsy remains the gold standard for distinguishing malignant tissue from benign one [163]. Despite the possibilities to make screening tests and monitor cancer patient's conditions using oncological markers, histology is needed to verify the diagnosis. Magnetic resonance imaging (MRI), computer tomography (CT), and positron emission tomography – computed tomography (PET-CT) are informative ways of cancer anatomical visualization, but they are costly and, during CT scan, expose the patient to relatively high radiation dosages.

The quality of diagnostics highly depends on the expert's qualification - the number of errors during the histopathological examination ranges from 4.87% to 11.8% [164]. In this regard, much attention is devoted to automatic algorithms of histological or tomographic image processing [165], but these methods' potential is still a matter of research. Here SERS presents itself as a powerful analytical method in tumor diagnosis.

### 7.1. Tumor localization and treatment

Even before SERS, Raman spectroscopy was considered to be a

promising method for the diagnosis of cancer [166]. As for SERS experiments [167–170], the most commonly used strategy uses Au NPs with a PEG coating. These structures show minor or negligible toxicity and provide high Raman signal amplification [167]. Moreover, they can be delivered directly to the tumor by passive or active mechanisms.

The passive approach is based on the tumors' enhanced permeability and retention (EPR) effect. This effect causes an increased accumulation of certain NPs inside the tumor due to its excessive angiogenesis and production of vasodilators and other substances. This effect is more prominent for NPs with a mass larger than 40 kDa [171]. Several groups used this mechanism to deliver their NPs to the cancer site without active targeting *via* antibodies or ligands [172–177]. However, one limitation is that only the primary tumor site is visualized, presumably, because of insufficient accumulation of NPs on the tumors' borders (or in secondary tumors) and, therefore, too low signal. One way to overcome this limitation is with gold star-shaped NPs covered by reporter molecules and $SiO_2$ on the surface to increase the signal enhancement. Those particles showed LOD of 1.5 fmol in *in vivo* and *ex vivo* experiments [172] on different tumors in mice including breast, pancreas, prostate cancer, and fibrosarcoma, with a resolution down to 100 μm (Fig. 10a). This example shows that initial limitations of SERS for *in vivo* applications could be overcome by increasing the enhancement factor of the nanoparticle system justifying the many reports on new plasmonic systems continuously introduced by hundreds of research groups worldwide.

The active mechanism involves the guided accumulation of SERS-active NPs in cancerous tissues due to nanoparticle's surface functionalization with antibodies to the cancer cell antigens or ligands to cancer cell receptors [178–187]. Such experiments were discussed in the review of Vendrell *et al.* [169]. Wang *et al.* [188] used the active mechanism of NP delivery for rapid intraoperative quantitative phenotyping to find residual carcinoma in tissue after lumpectomy. The localized effect of SERS allowed the visualization of individual normal and cancerous tissue fragments. Targeted NPs were associated with antibodies to the receptors EGFR and HER2 that are highly expressed in cancerous cells. Non-targeted NPs were also present as a negative control. The ratio was close to 1 and 2 for healthy tissues and tumors respectively. The peak intensity ratio allowed the authors to differentiate the tissue phenotype. The experiment was also performed with human breast tissue with anti-HER2-NPs as the targets. This method was performed on samples with an area of up to 4 $cm^2$ within 15 min, which makes it suitable for intraoperative use. Folic acid functionalization of SERS-active NPs is often used because cancer cells tend to overexpress folate receptors [184,185,189] (Fig. 10b). For example, Zhai *et al.* [184] used Ag NPs functionalized with β-cyclodextrin and folic acid to detect HeLa and 293T cancer cells and efficient monitoring of the antineoplastic drug dihydroartemisinin (DHA). After incubation with NPs, cells were passed through a microfluidic chip that allowed precise spectroscopy at the single-cell level. It was shown that a DHA concentration of 10 μmol or more induces significant intracellular changes.

It is important to notice that passive and active mechanisms are not mutually exclusive but work simultaneously. The passive mechanism highly depends on the NP mass and size, for example, the best tumor accumulation and excretion time from the body occurs for NPs with 60 nm in size [190]. Also, the presence of different functionalizing molecules affects the accumulation kinetics as observed for nanoparticles with transferrin that accumulate faster and more efficiently in tumors while those with a PEG coating show deeper infiltration in the cellular tumor mass.

Aside from tumor imaging, NPs are also used to combat cancer growth [174–176,178,180,181,191]. Besides a targeted delivery of antitumor drugs, NPs offer a way for photothermal therapy of cancer due to NPs heating caused by plasmonic excitation upon laser illumination.

SERS-active NPs are promising tools for theranostics since they are able to perform diagnostics and therapy functions simultaneously in a single platform. An excellent example of theranostic application is given by NPs with a $Bi_2Se_3$ core that enhance both the Raman signal and the heat generation [178]. NPs were functionalized with folic acid to improve cell permeability, and DNA and microRNA (AntagomiR-152) for cancer cell growth inhibition. The latter effect was achieved by microRNA release from the NP after absorption to the cell with the carrier DNA sequence that binds to oncomiRNA-152. Tumor diagnosis with SERS is based on the microRNA signal reduction while the therapy component comes from the genes' expression of proapoptotic proteins CHUK, CUL5, and GADD45A as well as the photothermal effect. These theranostic combinations used *in vivo* destroy up to 98% of cancer cells in 48 h

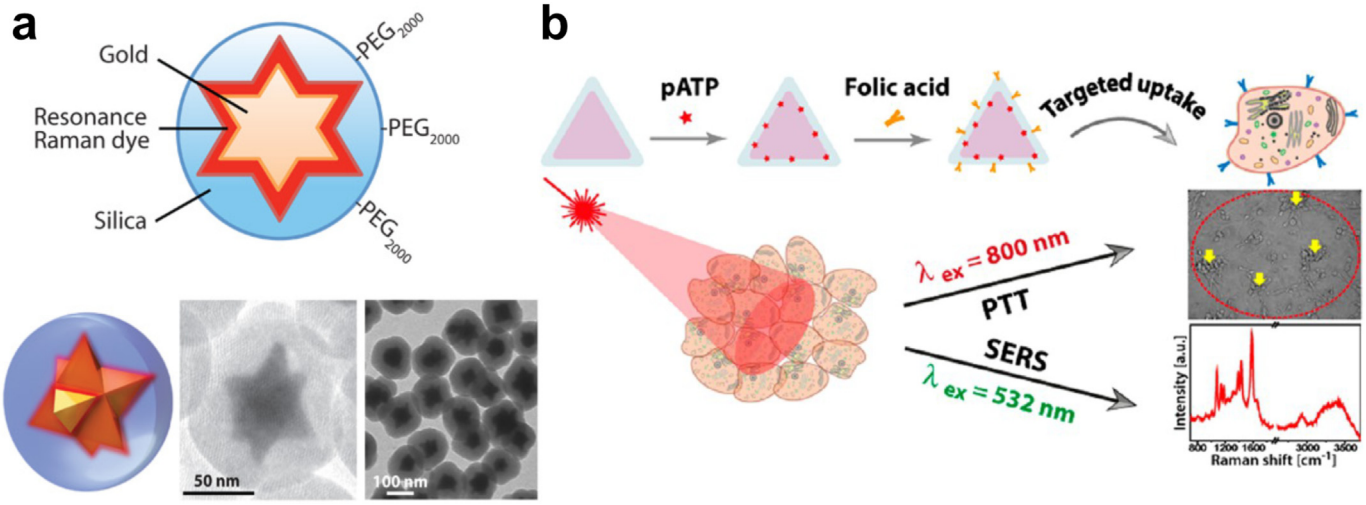



**Fig. 10.** (a) An example of NP for detecting tumors *via* SERS, aimed for passive accumulation in growth. Reproduced with permission from Ref. [172]. Copyright © 2015 Science Translational Medicine. (b) An example of NP for detecting the tumor *via* SERS, aimed for active accumulation in growth. Reproduced with permission from Ref. [189]. Copyright © 2014, ASC Publications.

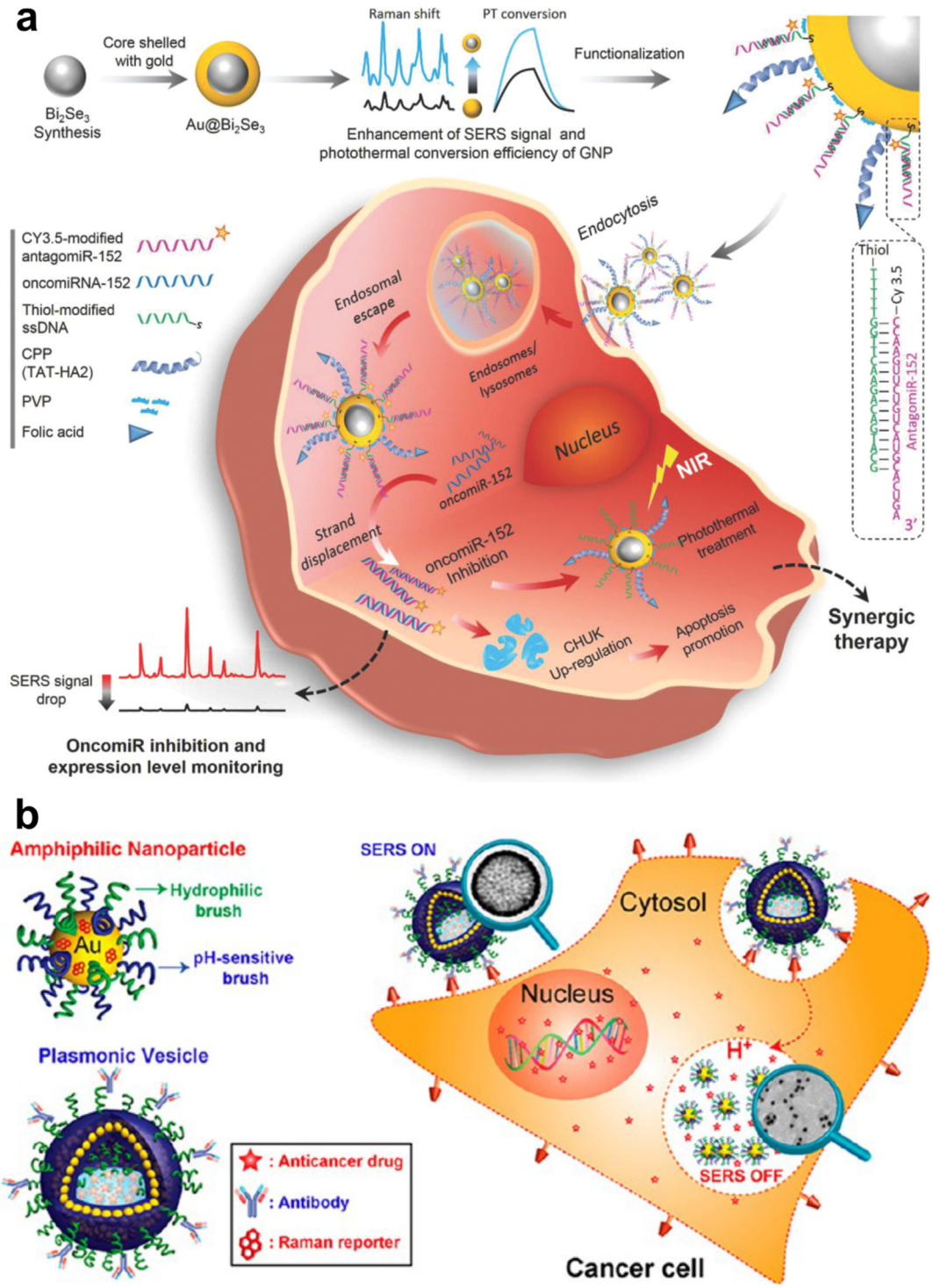



**Fig. 11.** (a) Principle of theranostic approach to cancerous cells. Reproduced with permission from Ref. [178]. © 2018 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim. (b) Self-assembly of Au NPs to the hollow vesicles containing antitumor drugs and their delivery inside the cells for the theranostic approach. Reproduced with permission from Ref. [180]. Copyright © 2012 American Chemical Society.

(Fig. 11a).

Targeted drug delivery can be achieved by putting doxorubicin into hollow vesicles, which were self-assembled by Au NP 14 nm in size with hydrophobic and hydrophilic (amphiphilic) protein layers and Raman reporters on its surface in aqueous conditions. An additional functionalization with HER2 antibodies makes these nanostructures penetrate cancer cells. The lysosomal acidic environment turns the hydrophilic protein into hydrophobic leading to the destruction of the vesicle. The Raman signal's disappearance indicates the cell's destruction while doxorubicin is released inside breast cancer cells as a therapeutic agent (Fig. 11b) [180].

A method that makes simultaneous detection and treatment possible is a rare but welcome occurrence in healthcare, especially

while dealing with cancer. SERS-active NPs can immensely ease the burden of side effects for the patient and the therapy complexity for the medical professionals. However, photothermal therapy of cancer is not a panacea and seems to be appropriate only for dealing with small growths. The rapid destruction of large amounts of tissue by heating inside the patient's body can lead to strong and potentially life-threatening inflammatory reactions. This problem worsens by the fact that most cancers get diagnosed at advanced stages [192]. Thus, the most probable application of the theranostic side of SERS seems to be the elimination of residual cancerous masses during and after surgical removal of a tumor. NPs should be paired with conventional methods, such as chemotherapy, for maximal efficiency of the therapy. This synergizes well with relative ease of turning the NPs into carriers for anti-tumor drugs without losing their SERS-active properties.

### 7.2. Liquid biopsy

Aside from the direct delivery of NPs to the cancer site, a different way of cancer diagnostics is liquid biopsy implemented by targeting specific oncomarkers in biological fluids, such as circulating DNA, antibodies for cancerous cell antigens, tumor metabolites, circulating tumor cells (CTC). Liquid biopsy is an essential tool not only for early or asymptomatic diagnostics but also for disease monitoring, relapse detection, and therapy efficiency control.

#### 7.2.1. Tumor DNA

One of the promising ways to detect cell death in the body is by evaluating circulating DNA released into the blood during apoptosis and necrosis. The increased nucleic acid blood level is related to genetic and epigenetic alterations inherent to cancer cells and is higher in cancer patients than in patients without tumors [193,194]. This offers an opportunity for disease monitoring and therapy optimization with SERS-based ultrasensitive detection of circulating DNA [195]. SERS provided such excellent sensitivity that even DNA changes at the single nucleobase level were visible in the spectral analyses of blood from 240 patients with half of them suffering from nasopharyngeal cancer [196].

Cancer DNA detection is greatly improved by combining SERS with different amplification methods, such as PCR, exponential strand displacement amplification, and amplification refractory mutation system. Fluorescent tags provide visualization and quantitative analysis of the molecules of interest although with lower spectral resolution than SERS due to significant fluorescence bandwidth (20–80 nm). This limits the use of only 4 different nanotags simultaneously in multiplex analysis [197]. In contrast, the SERS spectral bands have an approximate width of 0.1 nm, which vastly increases potential multiplex SERS analysis opportunities. The combination of SERS and machine learning algorithms for cancer detection [198] to photoinduced DNA damage [199] also showed the possibility for simultaneous detection of 8 different cellular metabolites *in vitro* at distances from 0 to 2000 μm from the cell's surface [200]. Results from primary human umbilical vein endothelial cells (HUVEC) and HeLa cervical cancer cells contain plenty of information on different metabolic pathways activity in healthy and cancer cells.

In this way, a combination of different amplification methods with SERS can remove the restriction on the number of target molecules that can be simultaneously detected. These combinations are achieved in different ways: by melting the target PCR product onto immobilized probes on SERS substrates, using amplification products as linking sequences to bind capture probes and reporter probes, or directly detecting fluorescence-labeled primers that contribute to signal magnification [201–203].

For instance, PCR and SERS make multiplex detection of 6 different mutations in BRAF, KRAS, and PIK3CA genes in samples from colorectal cancer patients possible [204]. Mutations in those genes can indicate the disease's clinical and pathological features and help selecting the right therapeutic approach. This combination was tested on 49 colorectal cancer patients' plasma samples and appeared to be easy to integrate into existing PCR processes. A similar experiment was performed by Wee *et al.* [205] who added a magnetic component, thus increasing the sensitivity further with DNA amplified by PCR. They used magnetic beads functionalized with SERS-active nanotags that increased the DNA concentration upon magnetic field application [205]. This technique enabled the detection of circulating DNA sequences, one from each of three different melanoma strains (Fig. 12a).

Different SERS-based methods can be implemented for circulating DNA detection. SERS enables us to solve several problems: provide high sensitivity, especially in combination with other techniques, and can detect several DNA mutations simultaneously. This last point is critical due to tumor heterogeneity which means that there can be various types of circulating DNA in blood from one cancer patient but different subclones from the tumor site. Therefore, revealing specific mutations and quantitative detection of respective DNA sequences more accurately provides the overall level of circulating DNA and can determine tumor type or tumor stage thanks to SERS.

#### 7.2.2. Modified nucleosides

Nucleic acids in the human body can undergo different enzymatic modifications. For example, RNA that fulfills its function can be destroyed by nucleases and then participate in new RNA molecule synthesis. After the completion of protein, biosynthesis RNA is often unavailable to participate in the resynthesis process, and thus it becomes the source of free modified nucleosides removed from the body with urine. Some of the modified nucleosides can be considered as general oncomarkers for different types of cancer [206,207].

Modified nucleosides in urine samples of healthy people and cancer patients extracted from urine by affinity chromatography were analyzed by SERS. Au NPs were added to the modified nucleosides and allowed the classification of the samples from patients suffering from nasopharyngeal, esophageal cancer and from healthy individuals with a sensitivity of 95.2%, 90.9%, 98.1%, and specificity of 97.2%, 98.2%, 95.7%, respectively [208]. The same method [209] allowed distinguishing samples from gastric cancer *vs.* normal, breast cancer *vs.* normal, and gastric cancer *vs.* breast cancer patients reaching sensitivity values of 84.0%, 76.7%, and 82.0%, respectively. The specificities for each combination were 95.8%, 87.5%, and 90.7%, respectively.

There are a few works devoted to modified nucleoside detection in biological fluids through SERS methods. However, the SERS study of modified nucleoside urine level is the most promising approach so far for distinguishing between cancer patients and healthy individuals which is critical in early diagnosis and cancer survival.

#### 7.2.3. Exosomes

The study of exosomes is one more important and interesting subject of research. These are small vesicles 30–100 nm in diameter secreted by many types of cells, including cancerous. Moreover, both internal and outer surface molecules of exosomes carry genetic information about their tissue of origin [210].

A prominent biomarker for exosome detection is CD63, which appears on the surface of all exosomes. This application is possible with MNPs that capture all exosomes from a test sample by binding to the CD63 aptamer [211]. Aptamer-functionalized Au NPs with markers for breast, prostate, and colorectal cancer and with a Raman probe for each of them were added to the sample. After

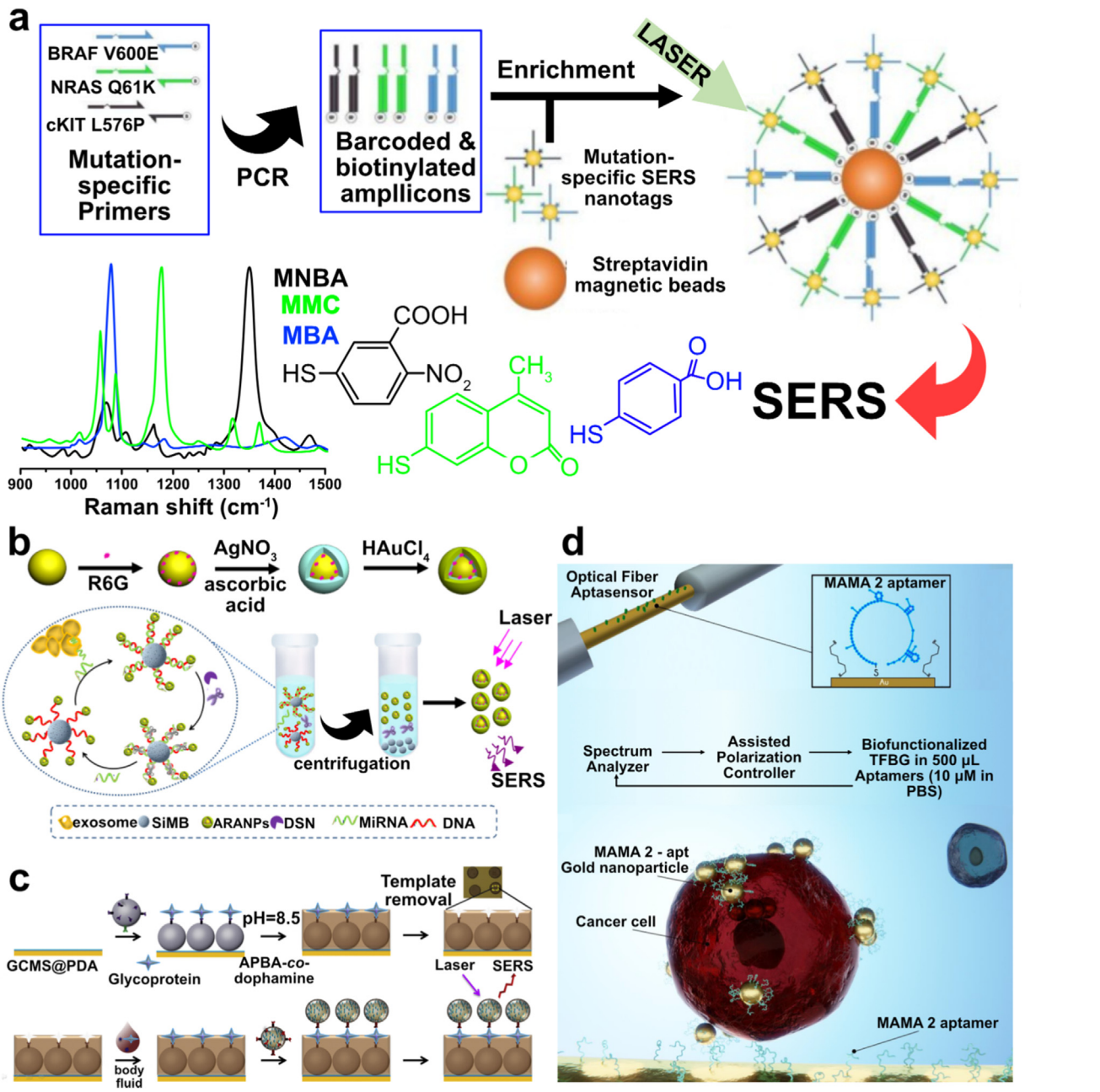



**Fig. 12.** (a) Applying SERS for detection of cancer DNA, preliminarily amplified with PCR. Adapted with permission from Ref. [205]. Copyright 2016, Theranostics. (b) Detection of exosomal miRNA with Au NPs with the complementary DNA sequence. Adapted with permission from Ref. [224]. © 2018 Elsevier B.V. All rights reserved. (c) Carcinoembryonic antigen evaluation by SIMP-method. Adapted with permission from Ref. [230] © 2019 Elsevier B.V. All rights reserved. (d) Detection of circulating tumor cells with optical fiber sensor. Adapted with permission from Ref. [241]. Copyright 2020, ACS Publications.

adding the desired biomarker, exosomes formed sandwich-like structures with magnetic beads and specific SERS-probes. A magnet could segregate these complexes, and the remaining liquid from the sample was analyzed by Raman spectroscopy. The presence of specific exosomes was evaluated by the reduction or complete signal disappearance of specific reporter molecules initially present in the sample. The LODs for the different exosome detection were 32, 73, and 203 exosomes/µL for breast, colorectal, and prostate cancer, respectively. This is a significant result since it also showed the possibility of differentiating three types of exosomes. This method's potential for clinical applications was shown in human blood experiments with those types of cancer as well as using other biomarkers [212–214].

The relatively big size of exosomes opens up simple and unique ways for their detection, such as large 3D porous structures, which act as a fishing net for exosomes [215]. Aside from the SERS effect, 3D inverse opal also enables the “slow light effect” that occurs when the laser beam gets trapped inside such a structure leading to multiple scattering and thus a multifold increase of Raman signal. This effect was evidenced with $TiO_2$ nanostructures coated with gold that allowed detecting exosomes from different cancers by enhancing up to two times the 1087 $cm^{-1}$ Raman peak related to P–O bonds.

#### 7.2.4. MicroRNA

Minor non-coding RNA molecules, microRNA or miRNA, as their name suggests, are small RNA molecules that participate in regulating gene expression by coupling with miRNA-targets suppressing the translation processes. Thus, miRNA participates in cellular proliferation, metabolism, differentiation, aging, *etc.* [216]. Malfunctions in regulation mechanisms involving miRNA contribute to the development of diseases including heart, kidneys, liver, and the immune system neurodegenerative disorders, cancer, *etc.* [217]. Those molecules are remarkably resistant against RNAses - enzymes that destruct RNA in blood - and thus could be a potential biomarker of diseases including cancer [218]. Again, the same principle with magnetic nanoparticles can be used here [219–221]. In all those experiments a complementary nucleic acid sequence was used to seek and capture miRNA. Of course, these capturing agents can be placed on nanoparticles or immobilized on SERS substrates for *in vitro* detection with LOD ranging from femto to attomoles [222,223].

Exosomes carry lots of different molecules, enzymes, proteins, and different RNA, such as miRNA. These molecular vehicles preserve some fragile components from the destructive effect of blood media [215]. Exosomes are present in various body fluids, such as blood, saliva, urine, breast milk [210]. It is also relevant to analyze not only exosomes' type and concentration but also to elucidate their internal structure and composition.

SERS is capable of exosomal miRNA detection from cancer cells and blood from non-small cell lung cancer patients [224]. This was performed by using sequentially assembled silicon microparticles with DNA sequence complementary to the desired miRNA and the SERS probe functionalized with rhodamine 6G (R6G) as the Raman reporter. The sample's miRNA formed a heteroduplex with the DNA of the SERS complex. This method's peculiarity is the signal amplification caused by using a duplex-specific nuclease (DSN) that breaks down the heteroduplex, and thus the extracted DNA can participate in a new cycle with the capturing agent. Once detached, the SERS-probe becomes available for detection after centrifugation. This amplification is critical because of the low levels of miRNA in the blood. miRNA-21 was chosen as the target molecule due to their overexpression in various types of cancer [225]. The possibility of distinguishing cancer cells from healthy ones by evaluating this marker was shown in cultures of non-small lung cancer cells (A549), HeLa, and normal kidney epitheliocytes. The capabilities of this method were demonstrated on samples from non-small cell lung cancer patients and healthy people with high performance comparable to real-time PCR. The LOD for miRNA was 5 fM thanks to a significant signal amplification due to DSN usage (Fig. 12b). A related strategy was implemented by Pang *et al.* [226,227] who achieved an impressive attomolar detection limit in the analysis of blood samples from healthy individuals and patients suffering from pancreatic ductal adenocarcinoma.

Thereby, the investigation of such informative objects like exosomes and miRNA through SERS-based methods is characterized by high sensitivity and specificity. This makes SERS a tool with great potential to complement current diagnostic approaches, especially for point-of-care applications. The multiplex detection of microRNAs, for which SERS methods could further enhance the analysis efficiency, remains still poorly explored. The development of detection systems for these biomaterials with new SERS platforms and analytical methods combinations has a strong but yet untapped potential for groundbreaking oncology research.

#### 7.2.5. Tumor antigens

An antigen is a macromolecule that contains information about foreign substances in the body, triggering an adaptive immune response. Tumors express a variety of antigens that are secreted into the tumor microenvironment. These molecules are represented by peptides, oligopeptides, phosphopeptides, *etc*., classified as shared tumor antigens and tumor-specific antigens, or neoantigens, that result from different somatic mutations. There are different immunoproteomic and genetic approaches for tumor antigen research [228,229]. SERS-based methods can also be exploited for this purpose.

The detection of carcinoembryonic antigen (CEA) in biological fluids was possible with SERS which is crucial since this is a marker for colorectal, breast, testicles, lung, stomach, bladder, and pancreas cancers. The benefits of SERS include simplicity and quick results for this biomarker detection that otherwise requires different immunoassays including radio-, fluorescent-, and immunochemiluminescent assays. For example, SERS with surface molecularly imprinted polymer technology (SMIP) provided better limits of detection than commercial electrochemiluminescent immunoassay in the analysis of CEA serum samples from healthy people and colon cancer patients (Fig. 12c) [230]. Several reports were dedicated to the determination of prostate-specific antigen (PSA) level in blood. This antigen is synthesized by prostate cells, and its concentration in the blood can elevate for prostatitis, benign prostatic hyperplasia (BPH), prostate cancer [231], and cancer relapse in patients who underwent radical prostatectomy [232]. Currently, the standard method for PSA detection involves different immunoassays, but combining it with SERS increases the sensitivity making LOD of 12 pg/mL for PSA in serum possible [233].

Besides immunoassays, MNPs with a plasmonic coating make once again an excellent combination for ultrasensitive analytical detection and therapy with SERS, this time in combination with antigens. MNPs with an Au coating and Au NPS both functionalized with PSA merge together in sandwich-structures that can be preconcentrated with a magnet to achieve a LOD of PSA of 0.75 ng/mL *in vitro* [234]. This LOD is several times better than PSA concentration in human serum associated with a high risk of prostate cancer (from 10 ng/mL) and so-called "grey zone" (4–10 ng/mL) when additional research methods must be used for the diagnosis. But that was not it, further analyses with cervical cancer cells showed good cell permeability by endocytosis, gave good contrast on MRI scans, and induced heating raising NPs temperature from 18 to 54 °C in 13 min causing cancer cells death by apoptosis.

In many cases, it is possible to significantly increase the method's diagnostic value by simultaneous detection of multiple markers [235,236]. This was possible with SERS that provided a high accuracy to distinguish and classify serum samples from healthy people, patients with benign prostatic hyperplasia, and patients with prostate cancer *via* simultaneous detection of PSA, prostate-specific membrane antigen (PSMA), and human kallikrein 2 (hK2). The SERS-based immunoanalysis of three oncomarkers, increased the diagnostic precision of prostate cancer from 50% to 70% and improved identification of benign prostatic hyperplasia patients and healthy people up to 60% and 75%.

SERS shows to be an excellent tool for simultaneous multiplex identification and characterization of plentiful tumor antigens. This can reinforce research on tumor immune profiling, cancer diagnostic, cancer vaccine development for cancer prevention, and selection of target immunotherapy.

#### 7.2.6. Circulating tumor cells (CTCs)

Besides cellular components, CTCs can be found in the blood. These cells are released from the primary cancer site and result in cancer or metastasis, which often leads to death. CTCs detection can be used for tumor diagnostics, selecting the best therapy, and monitoring and predicting the tumor response to different treatments.

The challenge to detect CTCs is their very low concentration

since even cancer patients usually have only 1–10 CTCs per 1 mL of blood [237]. There are some strategies to increase detection sensitivity including sample enrichment [238,239], the use of specific ligands to CTCs, such as aptamers [240,241], antibodies [242], and SERS CTC filters [243]. Different SERS approaches for CTC detection were reviewed previously [244]. For example, Loyez *et al.* [241] made a multiresonant optical fiber sensor, coated by a gold film, modified by aptamers for the breast cancer biomarker protein mammaglobulin A to accomplish an impressive detection level of 49 CTCs/mL in 5 min of spectroscopy. Their system was completed with the addition of Au NPs modified by the same aptamers for signal amplification – this improved LOD to 10 CTCs/mL (Fig. 12d). Despite low LOD in comparison with commercially available kits for CTC detection, SERS exceeds most of them in time of the analysis and cost [245].

Liquid biopsy is a rapidly developing branch of cancer diagnostics. New biomarkers are constantly being discovered and used in clinical practice. Most of them are present in blood and other biological fluids in vanishingly small amounts even during the advanced stages of cancer, so, most likely, only SERS with magnetic nanoparticles will take place in liquid biopsy. For further performance enhancement, a simultaneous analysis of multiple cancer markers should be performed.

### 7.3. Tumor heterogeneity

Cancer cells are characterized by a large heterogeneity, which manifests as differences in the cell's phenotype, metabolism, and function, even within the same tumor. This variability is reflected in the gene, transcriptomic, and proteomic levels. Tumor heterogeneity influences the growth, metastatic process, and therapy resistance of tumors. Thus, research and detection of cancer cell features at an individual level can open new perspectives for tumor treatment [246–251]. The cell nucleus is an abundant information source of tumor heterogeneity that can be accessed with SERS since we could confidently acquire this information by placing NPs near the nucleus. For this task [251] 40 nm Au NPs were conjugated with NLS peptide SV40 T large antigen and then added to culture media of human neuroblastoma cells for 72 h. SERS particles accumulated inside or near the cell's nucleus, making studying the concentration ratio of various nucleus components in differentiated and undifferentiated cells possible. PCA of SERS spectra showed that the differentiated cells have a higher expression of DNA peaks and histone proteins, attributed to a more compact laying of chromatin in these cells. At the same time, undifferentiated cells showed changes in DNA/RNA ratio, proving a lower chromatin condensation level. On the other hand, an important disadvantage of adding NPs into the cell culture medium is the irregular distribution of NPs in the medium and the cells themselves.

Tumor tissue does not consist only of malignant cells but it recruits healthy ones (fibroblasts, leukocytes, *etc.*) from surrounding media to provide nutrients and support for new growing tumor cells. Tumor cells are also not all the same - a non-ending battle between the most survivable cancer clones takes place in rapidly developing malignancies. Moreover, tumor heterogeneity influences susceptibility to treatment [252]. SERS studies of various cells in tumors can not only help cancer monitoring, but unveil numerous secrets of this highly dangerous disorder and help develop new methods - such as brand-new chemotherapeutic drugs, aimed not at cancer cell destruction, but rather auxiliary cells in the growth, thus disrupting the tumor growth or metastasis processes.

We have witnessed in this section the almost unlimited number of approaches that SERS offers for studying cancer, its diagnosis, and its therapy. Aside from tumor-related substances and CTCs measurements in biofluids, it is possible to gain critical information about the growth with or without SERS-active reporters by recording the spectra directly from cancerous tissue. Theranostic approaches appear now limited only by the imagination of researchers who are intensively developing these topics. Tumor occurrence is only expected to rise in the near future, so will the search for new ways of dealing with malignancies, and moreover - cancer remains a remarkably poorly understood foe. There are still ongoing debates about every aspect of cancer - its origin, progression and metastasis process, cellular composition, and mechanisms of treatment defense. Thousands of cancer-inducing genetic mutations remain undiscovered. New types of cancer, biomarkers, and tumor cells are discovered every year. Nearly every known method was thrown at the cancer frontier, and Raman spectroscopy is not an exclusion. It has all the chances to get its place in the rapidly expanding field of cancer research.

## 8. Infections

Infectious diseases remain a critical problem, causing the death of 10–16% of people worldwide according to World Health Organization (WHO) data from 2000 to 2016 [146]. Here, we describe novel detection methods of bacteria, protozoa, and viruses by Raman spectroscopy and SERS. It should be noted that each type of pathogen has its own set of diagnostics methods. Each of them will be briefly mentioned at the beginning of the corresponding subsection.

### 8.1. Bacteria

As of today, microbiological culturing of biomaterials from patients is the gold standard for diagnosing bacterial diseases. The critical drawback of this method is the time-consuming analysis process (24–48 h and longer). This is why most doctors administer broad-spectrum antibiotics before bacterial culture microscopy results from the patient arrive. This therapy can lead to emerging of resistant bacteria strains. Although PCR analysis can be a viable tool for bacteria diagnosis since it requires a much shorter time (2–3 h), it cannot detect pathogens for which DNA is not present in the primers kit [253]. This limitation occurs in many ultrasensitive SERS combinations described in this review, but the label-free intrinsic nature of SERS allows detecting molecular analytes present in bacteria without pre-knowledge. Notice that microbiological culturing often measures its results in colony-forming units (CFU) - a number of bacterial cells enough to form one colony on the plate.

Raman methods can be helpful in bacterial infection research though they have not received much attention [254,255]. Opportunities for SERS applications to identify bacterial diseases [256–258] are rapidly developing because of its fast measurement capability and minimal sample preparation. SERS-based approaches can also recognize the strain of pathogenic bacteria and its resistance to antibiotics, which otherwise require additional analyses if performed by conventional methods.

SERS offers high flexibility for researchers, as it is possible to put the nanoparticles at any point near the bacteria. At the same time, lateral-flow immunoassay is a perfect combination for SERS implementation [259,260] as shown with DNA primers complementing the highly specific nucleic acid sequence of *Listeria monocytogenes* and *Salmonella enterica* added to those bacteria lysate in a functionalized strip [259]. After adding the primers to the strip, Au NPs conjugated with streptavidin got released from the paper and bound to biotin providing visual and instrumental verification of results with a LOD of 19 and 27 CFU/mL for listeria and salmonella, respectively (Fig. 13a).

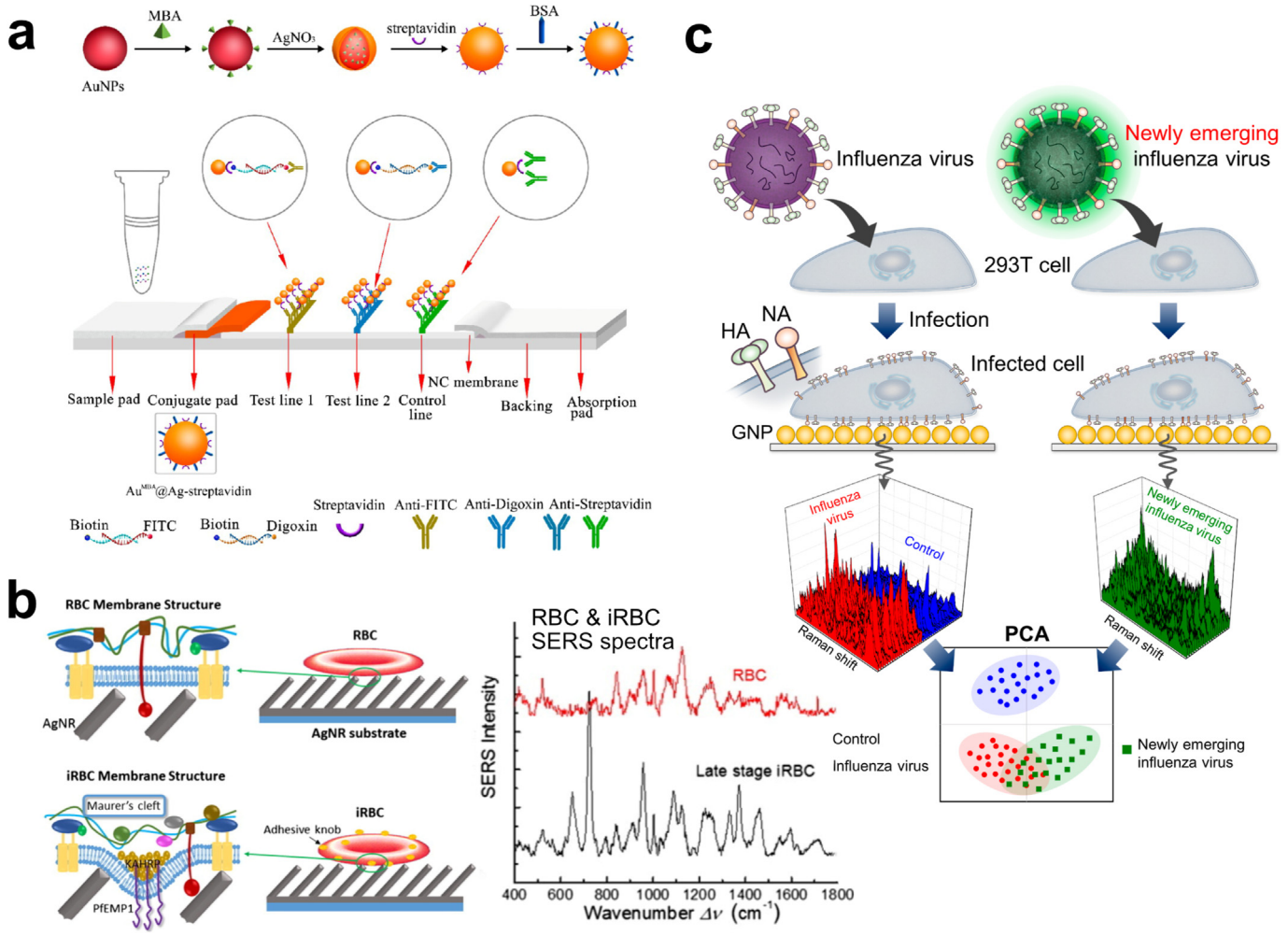



**Fig. 13.** (a) SERS-Based Lateral Flow immunoassay for detection of DNA of bacteria. Reproduced with permission from Ref. [259]. Copyright 2017, ACS Publications. (b) Detection of malaria plasmodium species inside red blood cells on a silver substrate. Reproduced with permission from Ref. [269]. Copyright 2016, Elsevier. (c) Viral replication evaluation in cancer cells on a gold substrate. Reproduced with permission from Ref. [278]. Copyright 2019, ACS Publications. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Thanks to its simplicity, the application of colloidal NPs directly on bacteria's surfaces is becoming widespread [261–263]. Some of those methods are based on the attachment of positively charged particles to the bacterial wall with negative zeta-potential to detect bacteria in different media like in drinking water with, showing a LOD of 2.5 × $10^2$ bacteria/mL [261]. A microfluidic device with a hollow-core photonic crystal fiber and a flow of Ag NPs inside microchannels [262] provided a LOD of up to 4 CFU/mL in 15 min of measurement, sufficient level of reproducibility (5–15% of differences of the results between the measurements), and good correlation with microbiological culture ($R^2$ = 0.96). One of the limitations of this approach is that in some cases Ag NPs could lead to partial cell death and, therefore, to classification errors.

Another common configuration often discussed in this review is combining these methods with MNPs [264–266] for bacterial infection. When MNPs get attached to the bacteria's surface as in previous analysis, pre-concentration becomes straightforward with a simple magnet and thus increases the Raman signal. By using this strategy, *Escherichia coli*, *Staphylococcus aureus,* and *Pseudomonas aeruginosa* were detected in culture media and infected whole blood samples with a LOD of 10 CFU/mL [265].

The scientific search for the place of SERS in bacteria research focused on diagnostics and antibiotic resistance tests. However, another interesting topic here is monitoring the vital activity of bacteria, their biochemical processes, especially inside the living bacterial cell. It would be fascinating to see experiments where one could manage to put NPs in the bacterial cytoplasm. Naturally, there are significant challenges to accomplish this task - the small size of bacteria and thickness of cell wall are the most obvious ones, requiring extremely tiny NPs and new ways of their delivery. This could shed light on some biological mechanisms of prokaryotic cells that remain a mystery.

### 8.2. Protozoa

In 2019 alone, there were 229 million malaria cases, of which 409 thousand were fatal. 51% of the cases were registered in underdeveloped African countries - Nigeria, the Democratic Republic of the Congo, Uganda, Mozambique, and Niger [267]. The gold standard of malaria diagnostics is light microscopy, which offers the doctor rich information, including malaria species, stage of disease, and presence of other parasites in case of mixed infection. However, this method is dependent on equipment quality and staff qualification. Therefore, the development and implementation of fast, reliable, and inexpensive methods of diagnostics, such as SERS, would be a desirable choice for African medics. Rapid diagnostics

tests based on immunochromatography are already present, but they are not as informative as microscopy [268]. Moreover, except for malaria, diagnostics and research of protozoa-induced infectious diseases with Raman spectroscopy and SERS are poorly represented in the literature [266,269,270].

*Plasmodium falciparum* DNA can be detected in blood lysate by hybridizing the target DNA sequence of the parasite with two probes containing complementary sequences [266]. One probe was functionalized with the complex Au NP and another one with a magnetic bead. This structure then went through multiple washing cycles in a magnetic field to remove non-hybridized nucleic acid sequences and reduce parasitic signals. Without the need for gene amplification, an impressive LOD of 2 amol on a 10 μL sample was achieved. The same parasite was detected with SERS in infected blood samples at different stages of protozoa development using Ag nanorods [269]. Direct spectral comparison between contaminated and normal erythrocytes allowed detecting the parasite after 16 h of incubation and determining its development stage (Fig. 13b). Perhaps the most unique approach so far used gold-coated *G. weiskei* butterfly wings as SERS substrates, allowing parasite detection in 0.005–0.0005% concentrations from the infected erythrocyte lysate [270].

Besides malaria, there are other problematic protozoan diseases, including sleeping sickness and dysentery caused by trypanosomes and amoebas, respectively. As mentioned earlier, Raman and SERS implementation to deal with these infections are not broad enough. In addition, protozoan diseases burden healthcare services in developing countries [271], with outbreaks periodically happening in developed countries, such as the USA and New Zealand [272]. Thus, there is a need and research opportunity on the development of SERS-based methods for protozoa detection.

### 8.3. Viruses

SERS is also useful for virus detection in biological fluids. As the unfolding COVID-19 pandemic is showing us, viruses are a rapidly changing lifeform, requiring the emergence of new methods for their diagnostics to match the viral mutation speed [273]. SERS offers significant benefits that make it a strong contender in this competition.

The general principles of SERS viral detection are similar to those of bacteria detection - some of them use colloidal NPs [274] (including the methods with magnetic particles [275]) and SERS substrates [276–278]. For example, an aptamer-functionalized Ag surface allows the detection of a whole influenza virus in allantoic fluid [276]. After virus immobilization, free aptamers linked to Raman-active dyes (Cy3 or Bodipy FL) induced the formation of sandwich-like layer “aptamer-virus-aptamer” structures. This approach was applied to detect ten different influenza virus strains at high levels of selectivity and sensitivity with LODs of $2 \times 10^4$ and $1 \times 10^4$ for Cy3 and Bodipy FL, respectively in 250 μL of the sample.

Research and diagnosis of new influenza viruses can be done directly with embryonic kidney cells (293T) cultured on a SERS-active substrate [278]. Each virus strain forces the cells to synthesize unique proteins that have characteristic Raman spectra and thus it allows detection due to viral gene expression (Fig. 13c).

Lateral flow immunoassays combined with SERS for virus detection are fairly popular in the scientific community [279–282]. In this method, the virus is attached to the antibody-functionalized test strip, and then Au NPs are deposited to enhance the SERS signal. Magnetic $Fe_3O_4$ NPs in a SERS-active substrate concentrate the virus from a sample reaching LODs of 50 and 10 PFU/mL for H1N1 virus and human adenovirus [280]. Bovine leukemia virus antigen gp51 was also detected in a similar way [281]. Magnetic gold nanoparticles were used with DTNB Raman-labeled gold nanorods and anti-gp51 antibodies to capture gp51. These features combined with oriented antibody immobilization led to improved LOD and limit of quantification (LOQ) of 0.95 mg/mL and 3.14 mg/mL, respectively.

Here, we showed possibilities offered by SERS and Raman spectroscopy to evaluate any kind of pathogens from protozoa to viruses - with high efficiency. These approaches allowed to distinguish several diseases with high social importance, from Ebola and Lassa viruses to malaria pathogens in infected blood samples with high sensitivity and specificity [285], and to detect traces of West Nile and Rift Valley viruses and *Yersinia pestis* in fetal bovine serum directly and without time-consuming sample preparation procedures [286], which is desirable in time-sensitive situations and real-time monitoring of patients.

A remarkable approach with dual SERS nanotag/substrate platform functionalized with antibodies allowed norovirus (NoV) detection based on plasmonic/magnetic molybdenum trioxide nanocubes (SERS nanotag) and chemically active graphene-based substrate conjugated with 4-MBA (SERS substrate/reporter) [282]. NoV-like particles detection showed a LOD of ~5.2 fg $mL^{-1}$ and ~60 RNA copies/mL in human fecal samples, $10^3$-fold better than the commercially available ELISA kit for NoV. This method's flexibility brought by MNPs enables the creation of digital microfluidic (DMF) chips. In this [283] chip configuration, the different components of the SERS immunoassay are added by successively moving magnetic beads inside the chip to the compartments with specific reagents. As a result, DMF greatly reduces immunoassay's components consumption and incubation time and enhances the effectiveness of washing, a highly important procedure in all immunoassays.

In many cases, it is crucial to know the virus genotype. For example, we can roughly divide human papillomavirus (HPV) strains into low-risk and high-risk types. HPV 16 and 18 from the latter group cause 70% of cervical carcinomas worldwide. Thus, genotyping is performed to evaluate viral strain. Hibbitts *et al.* [284] implemented SERS as a commercial SERS HPV genotyping assay RenDx. After PCR, the DNA probes functionalized with Raman dyes are added to the PCR products. Hybridization takes place if the sample's DNA material contains desirable sequences complementary to probes. After that, hybridized strands are placed on streptavidin magnetic beads and washed from unhybridized or reagent leftovers. Then probes are removed from DNA sequences and added to silver nanoparticles suspension, and Raman spectroscopy occurs. The signal from Raman dyes tells about the presence of specific HPV strains. This assay allows screening six different strains simultaneously, including four high-risk types. Although very promising, 2 out of 25 experiments failed to detect HPV 16, which shows that significant progress is still needed.

The COVID-19 pandemic brought massive attention to the various virus detection methods, where they showed themselves as an efficient diagnostic tool with their limitations. Immunoassay and antibody-based rapid diagnostic kits work only after the SARS-CoV-2 (severe acute respiratory syndrome-related coronavirus 2) has gone from the patient's organism, as it searches for antibodies, which appear only by the end of the illness. PCR is a widely used method for diagnosing currently ill individuals by finding viral gene sequences, but this method suffers from a lengthy analysis time (up to 3 h) [18]. Being a fast and universal method, SERS could be an exciting and critical addition to the arsenal of physicians in the early diagnosis of viral outbreaks.

## 9. Outlook and conclusions

Raman spectroscopy and SERS are a powerful addition to the arsenal of medical practitioners, but yet its potential remains

**Table 2**
Methods of diagnosis of pathological processes: standard methods and implementation of Raman spectroscopy.

| Pathological processes | Classical analysis methods | Raman spectroscopy | *in vitro* SERS | *in vivo* SERS |
|---|---|---|---|---|
| Necrosis/ Apoptosis | NMR, CT, X-ray diagnostics [290], ELISA [291] | ✓ |  | ✗ |
| Inflammation | ELISA, immunoturbidimetry, agglutination tests [292], isoelectric focusing together with western blot analysis, LC-MS, MALDI-TOF MS [293], CLIA [294]. | ✓ | ✓ | ✓ |
| Hypoxia | Pulse oximetry [295], blood gas analysis [296], cardiotocography [297] | ✓ | ✓ | ✓ |
| Tumor | Histology, MRI, CT, X-ray diagnostics, PET, medical ultrasound imaging, ELISA [163,298], liquid biopsy [299] | ✓ | ✓ | ✓ |
| Infection | ELISA, PCR, microscopy, microbiological culture, chemiluminescence immunoassay (CLIA), matrix-assisted laser desorption/ionization with the use of Time Of Flight Mass Spectrometry (MALDI-TOF MS) [253,300] | ✗ | ✓ | ✗ |

untapped. SERS is on par with conventional methods in terms of specificity and selectivity but far exceeds them in the detection limit, allowing us to register single molecules of different substances. In Table 2, we show the standard methods for clinical analysis of different pathologies and contrast them with the application of Raman and SERS-based analyses developed until now. Raman spectroscopy brings inexpensive, label-free, and reliable detection of various pathological processes, which accompany many severe conditions, including cancer, cardiovascular diseases, and infectious diseases. The addition of SERS (and MNPs) presents to the researcher an unprecedented level of flexibility in working with molecules, organelles and cell compartments, whole cells, tissues, and others thanks to the high level of spatial localization of SERS, especially with confocal Raman microscopy imaging. The NPs themselves feature theranostic properties, which can be further enhanced by functionalizing with drugs, enzymes, and other molecular linkers with high affinity to the target site. This property is particularly highly desirable while dealing with cancer. SERS performance is comparable with most other instrumental methods of chemical analysis, such as ELISA, PCR, and chromatography, boosting their analytical performance when working in tandem. As of today, Raman spectroscopy has already become a powerful tool in the hands of scientists, but it still has to overcome some limitations to take a steady place in clinical laboratories. One of the crucial steps that have yet to be achieved by Raman spectroscopy is the sufficient availability of the method. Although the cost of analysis is relatively low and fast, the price of Raman spectroscopy devices is still high (~$50000 for a handheld model and ~$100000 for a benchtop [287]). To make Raman spectroscopy more widespread, it could also be implemented in modern non-invasive, portable, and real-time devices for different substance detection. For example, several works were dedicated to developing *in vivo* methods for blood glucose levels [288,289]. The potential application of Raman spectroscopy should not be limited to lab technicians, but surgeons should also profit from this method. Potentially helping perform real-time *in vivo* monitoring of different organs' conditions at the biochemical level, or precisely localizing the region of impaired tissue during surgery. Besides Raman making so much information accessible to clinicians, another important factor that must be established in the future is the safety of different SERS-active tags and reporters. After these cost and safety challenges are overcome or shadowed by the benefits SERS provides, we expect an accelerated translation of this method into clinics leading to a bright future for SERS in the medical field.

A critical challenge of *in vivo* biochemical analysis is the need for extreme sensitivities, the fact that some molecules are very similar, and the complex "dirty" environment of realistic physiological fluids. SERS is rising to these challenges with multiplexed sensing and magnetic nanoparticles that provide amazing LODs while selectivity is achieved by functionalization with naturally existing lock-and-key mechanisms of living systems. In many cases, the LOD achieved by SERS is far better than that of conventional methods. In turn, by using SERS, it will be possible to study earlier stages of pathological processes and better understand prevention, therapy, and remission. These characteristics alone can profoundly impact healthcare since we cannot base a diagnostic method on something that cannot be measured. After a new method shows the possibility of studying biomarkers at ultralow concentrations, it becomes a tool medical practitioners could use for improving patient well-being that is the ultimate factor deciding the implementation of new technologies in medicine.

## Declaration of competing interest

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.

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