Label-free surface-enhanced Raman spectroscopy of biofluids: fundamental aspects and diagnostic applications

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Abstract In clinical practice, one objective is to obtain diagnostic information while minimizing the invasiveness of the tests and the pain for the patients. To this end, tests based on the interaction of light with readily available biofluids including blood, urine, or saliva are highly desirable. In this review we examine the state of the art regarding the use of surface-enhanced Raman spectroscopy (SERS) to investigate biofluids, focusing on diagnostic applications. First, a critical evaluation of the experimental aspects involved in the collection of SERS spectra is presented; different substrate types are introduced, with a clear distinction between colloidal and non-colloidal metal nanostructures. Then the effect of the excitation wavelength is discussed, along with anomalous bands and artifacts which might affect SERS spectra of biofluids. The central part of the review examines the literature available on the SERS spectra of blood, plasma, serum, urine, saliva, tears, and semen. Finally, diagnostic applications are critically discussed in the context of the published evidence; this section clearly reveals that SERS of biofluids is most promising as a rapid, cheap, and non-invasive tool for mass screening for cancer.

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Introduction

Raman spectroscopy (RS) is a well-established analytical technique [1–5] based on the inelastic scattering of monochromatic light (i.e. a laser) by the vibrating atoms constituting the sample under investigation. Surface-enhanced Raman spectroscopy (SERS, also known as surface-enhanced Raman scattering [6–9]) is a vibrational-spectroscopy technique based on the intensity amplification of Raman scattering by metallic nanostructures with suitable plasmonic characteristics (e.g. metal nanoparticles). The close correlation between a Raman spectrum and molecular structure, and the ease of use and portability of this technique, are the basis of both qualitative and quantitative analytical applications of RS, ranging from chemistry and physics to materials and environmental sciences, forensics, biology, and medicine. Despite its advantages, however, RS intrinsically suffers from poor sensitivity, typically detecting analytes having at least molar to millimolar concentrations. Such poor sensitivity is hardly a limitation when studying solid samples or pure liquids, but it becomes a serious restraint when analyzing solutions in which analytes have micro to nanomolar concentrations, a condition that is frequently met in bioanalytical and biomedical applications. The intensity amplification of Raman spectra produced by SERS has been regarded as the solution to the sensitivity problem of RS. However, although SERS was first reported in the 1970s, it is only in the last 10 years [10], concurrently with the development of nanotechnology and plasmonics, that it has rapidly developed as a promising technique, applied to a variety of different fields by a widespread and interdisciplinary community.

Although SERS was initially limited to dilute aqueous solutions of relatively simple systems (e.g. small molecules and molecular ions), recent advances in nanotechnology, plasmonics, and photonics encouraged the application of SERS to biological systems of increasing complexity, including macromolecules, cells, tissues, and, of course, biofluids, the topic of this review. Biofluids are rich in information that can be exploited for therapeutic or diagnostic purposes. Qualitative or quantitative detection of one or more analytes with SERS can be performed either directly or indirectly, using two completely distinct approaches (Fig. 1). When SERS detection is direct (i.e. "label-free" approach [11]), the observed bands are associated with the analytes themselves, whereas in the indirect approach, the spectroscopic signal arises from one or more "SERS labels", i.e. chemical species (usually dyes) having an intense, stable, and well-recognizable SERS spectrum used to signal the presence of the analyte of interest [9, 12–14]. The systems constituted by such labels adsorbed on one or more metal nanoparticles are often referred to as SERS "labels", "markers", or "tags" and are the basis of SERS immunoassays, qualitative or quantitative tests to detect specific disease markers using antibodies [15–20]. SERS immunoassays, because of their sensitivity and their multiplexing potential (i.e. the possibility of simultaneously detecting more than one analyte in a single test), have gained increasing popularity for biofluids diagnostics, and have been recently reviewed elsewhere [17, 18, 20, 21].

Instead, this review focuses on the more fundamental aspects of the direct, label-free SERS of biofluids, and in particular on its applications in diagnostics. In the label-free approach, extremely complex aqueous solutions, for example human biofluids, freely interact with a nanostructured metal surface, the properties of which can vary substantially between different SERS substrates. Depending on the processes occurring during such interactions, SERS spectra may or may not be generated. Because of the complexity of such systems, basic aspects, including the interaction between the metallic SERS substrates and the biomolecules constituting a biofluid, need to be investigated. The effect of the SERS substrate, its interplay with the excitation wavelength used, the number and type of biomolecules which freely adsorb on such metal surfaces, their relation with the bands observed, and the meaning of the spectra when applied to diagnostics are all examples of questions to be answered.

The answers to these questions are partially found in the literature, and are critically analyzed in this review, the first part of which deals with some experimental features of SERS which are relevant for biofluid analysis. A description and, wherever possible, a "biochemical" interpretation of the SERS spectra for each biofluid will be given in the second part of this review. Current understanding of the fundamental aspects of SERS spectra of biofluids, as described in the first two parts, is the rationale for the application of SERS to diagnostics, and this is described in the last part of this review.

SERS: experimental aspects relevant to biofluids

Both theoretical and experimental aspects of SERS have been thoroughly described in both reference books [6–9] and recent reviews [10, 22–25]. The reader should refer to these works for the basics of SERS. However, despite the huge amount of

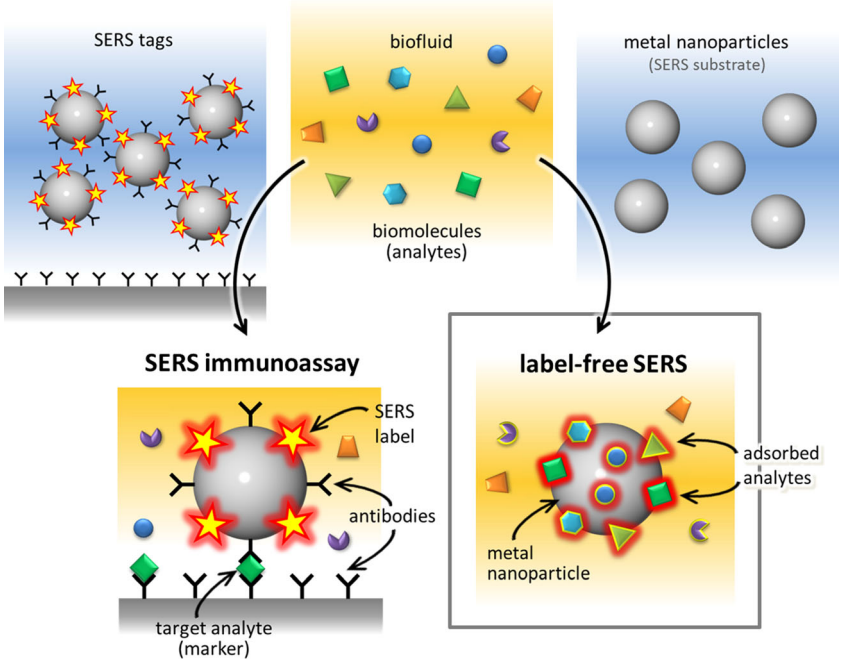

Fig. 1 Diagram schematically showing the differences between a label-free SERS approach and a SERS immunoassay using labels. In label-free SERS, the spectroscopic signal results from all those analytes which adsorb on the SERS substrate (direct detection), whereas in the SERS immunoassay the spectroscopic signal results from the labels on a SERS tag (indirect detection) that specifically binds to a target analyte

experimental evidence accumulated in decades of literature, many important aspects of SERS, both theoretical and experimental, are still often misunderstood, as pointed out by Martin Moskovits in a recent paper [26]. The intensity and shape of a SERS spectrum strictly depend on the combination of many experimental conditions, which should be adapted on the basis of the physico-chemical characteristics of the sample under investigation.

When considering such complex chemical mixtures as biofluids, with a variety of pH, ionic strengths, and optical properties, and having thousands of components with a wide range of concentrations, a solid understanding of the basic experimental aspects of SERS is essential to obtain meaningful data from experiments. An excellent introduction to the most important experimental aspects of SERS in general can be found in Ref. [27]; in this review we will rather focus on some aspects which are particularly relevant for SERS of biofluids.

Types of SERS substrate

There is a wide variety of SERS substrates [28–33], but for our purposes they can be divided in two classes: aqueous colloidal dispersions of metal nanoparticles (i.e. metal sols or colloids), and all the rest (i.e. non-colloidal substrates). The latter include both metal-nanoparticle aggregates, deposited and/or self-assembled onto a solid support (e.g. silicon, glass, paper) by bottom-up approaches, and ordered metal nanostructures obtained by chemical or physical top-down processes (etching, nanolithography, etc.).

Because individual metal nanoparticles have a negligible SERS effect [26, 34–36], whereas nanoparticle aggregates have a strong signal enhancement [37, 38], SERS spectra are observed only when metal colloids aggregate upon addition to the biofluid. Metal colloids are stabilized by electrostatic repulsions resulting from charged species (e.g. citrate or chloride ions) adsorbed on the nanoparticle surfaces [39–41]. Aggregation can be induced by the reduction of such repulsive forces upon displacement of these charged species by non-charged molecules (i.e. analytes which are chemi or physisorbed on the metal surface), or, consistently with the DLVO theory of colloidal stability [41], by an increase of the ionic strength of the dispersing medium. Electrolyte concentration can vary greatly between different biofluids [42], but in principle it is usually high enough to induce aggregation of metal colloids. However, many proteins, in particular serum proteins, are known to readily adsorb on metal nanoparticles, forming a "protein corona": a thick protein layer which hinders aggregation by steric repulsion, even in the presence of high ionic strengths [43–45].

Therefore, the presence of proteins is an important aspect to consider in SERS spectroscopy of protein-rich biofluids, for example blood, serum, and plasma, if metal colloids are to be used as substrates [46]. Colloid-based SERS spectra of protein-containing serum or plasma were reported after addition of aggregating agents [47, 48] and for dried samples (i.e. after incubation of the serum–Ag colloid mixture at room temperature for 1 h [49–51]). However, all efforts in our lab to obtain aggregation of metal colloids, as obtained from synthesis, upon addition to serum or plasma were frustrated, even after several hours of incubation at 4 °C [46]. In contrast, addition of metal colloids to plasma or serum samples filtered at 3 kDa, from which most proteins were thus removed, causes immediate aggregation, enabling the observation of intense SERS spectra [46]. Such an aggregation can be easily observed by eye as a color change of the colloid–biofluid mixture from red or yellowish to dark blue-grey.

The use of concentrated Ag and Au colloids with a higher nanoparticle density, obtained by centrifuging and resuspending the nanoparticles in a smaller liquid volume, apparently helps in overcoming the aggregation problems caused by the serum proteins. Casella et al. reported obtaining SERS spectra from blood by use of concentrated Ag colloids [52]. SERS spectra obtained with both concentrated Ag and Au colloids were also reported from both serum and plasma, upon incubation of the biofluid–colloid mixture for 1.5 to 2 h at 4 °C [53–57]. These observations are consistent with the fact that, because the number of nanoparticle aggregates in the probed volume is proportional to the SERS signal, a higher nanoparticle density will yield more intense SERS spectra. However, the mechanism by which aggregation of concentrated colloids can take place in the presence of serum proteins remains to be clarified. A partial aggregation already occurring upon centrifugation (i.e. before the addition to the biofluid) cannot be excluded, and it would explain the observation of SERS spectra even in the presence of serum proteins.

Other biofluids apparently do not suffer from similar aggregation problems, at least not to the same extent as blood. Protein content in urine is low [58], and SERS spectra of urine using metal colloids have been reported [59–61]. However, several pathological conditions greatly increase protein concentration in urine [58], and colloidal stability caused by proteins should be taken into account for those diseases. In tears and saliva, on the basis of the few reports available [62, 63], the proteins do not seem to hinder aggregation in metal colloids used as SERS substrates.

When non-colloidal substrates are used, including those prepared from the deposition of metal colloids onto solid supports, aggregation is not a problem. SERS spectra of unfiltered blood, serum, and plasma obtained using such non-colloidal substrates have been reported [47, 64, 65]. The physico-chemical characteristics of the substrate surface will also affect which analytes present in a biofluid will adsorb on the metal, thus appearing in the SERS spectrum. This holds true for both colloidal and non-colloidal substrates. For instance, negatively charged surfaces will hinder the adsorption of

species with negative charges, which, however, will readily adsorb onto positively charged substrates [66] (and vice versa). In general, it should be clear that SERS is a surface technique, and thus it is not neutral with respect to the sample. A SERS spectrum is always the result of an interaction between sample and substrate, so that, for the same biofluid sample, substrates with different characteristics will yield different spectra.

Selection of excitation wavelength

A careful choice of the laser wavelength is crucial when acquiring SERS spectra of biofluids. In particular, two aspects must be taken into account:

1. The laser wavelength must be able to excite the collective motions of surface electrons (i.e. plasmons) of the SERS substrate used, as required by the electromagnetic theory of the SERS effect, which accounts for most of the intensity enhancement produced [8, 9, 26];
2. The occurrence of a possible "resonance Raman" (RR) effect: the laser wavelength might be in resonance with electronic transitions of some analytes present in the biofluid, enhancing the intensity of the bands of those analytes over the bands of others and interfering with their observation.

If both RR and SERS effects take place at the same time, they combine to produce huge intensity enhancements for the bands of the resonant species, in a synergic effect called surface-enhanced resonance Raman scattering (SERRS).

Concerning the first aspect, a mistaken belief is the assumption that extinction (i.e. absorption plus scattering) spectra of SERS substrates are an indication of the wavelength to be used for laser excitation. In the past, it was believed that an optimum SERS effect could be obtained by using a laser with a wavelength falling in, or close to, an extinction band, corresponding to a surface-plasmon mode. When using metal colloids, excitation was believed to be optimum when matching the extinction band observed for aggregated nanoparticles. However, recent studies [67] established that there is no direct correlation between the extinction spectrum and the SERS wavelength response. This apparent discrepancy was solved by proving the occurrence of so-called "dark modes", i.e. plasmon-resonance modes which are not detected using far-field excitation, as opposed to the "bright modes" observed in the extinction spectrum [67, 68]. Indeed, intense SERS spectra were obtained from biofluids using Ag colloids and a 785 nm laser [46, 49, 53, 59, 69], even though such an excitation wavelength is very far from the extinction band for those substrates (approximately 400–410 nm for unaggregated Ag nanoparticles). Moreover, because dark modes arise from plasmon hybridization in interacting nanoparticles, they can also occur in non-colloidal substrates when these are formed by nanoparticles deposited on a solid support [67, 68]: SERS spectra of serum on Ag nanoparticles deposited on glass were obtained using a near-infrared excitation at 785 nm [47], despite the extinction maximum for those nanoparticles being reported at 418 nm.

As far as RR effects are concerned, biofluids contain many biochemical species absorbing in the visible region of the spectrum. For instance, blood contains hemoglobin and carotenoids, both absorbing in the blue–green: the hemoglobin present in erythrocytes is responsible for the bright red color of blood, whereas carotenoids are responsible for the yellow-orange color of blood serum or plasma. Hemoglobin bands dominate SERS spectra of whole blood, and beta-carotene bands dominate SERS spectra of serum and plasma, when using excitation in the blue–green [46, 52], indicating the occurrence of a RR effect. In some circumstances the RR effect is deliberately sought in SERS experiments, to selectively study those specific biomolecules even when present in complex environments (e.g. hemoglobin inside erythrocytes [70, 71]). But in many cases, RR bands are just interfering with the signal of other analytes, and are therefore a problem. However, when using excitation in the near-infrared (e.g. 785 nm) no RR effect takes place, and no hemoglobin or carotenoid bands are observed in SERS spectra of whole blood, serum, and plasma [46, 64]. No systematic studies are available on the possible occurrence of RR in other biofluids, many of which are slightly colored (e.g. urine). However, the cases of blood, serum, and plasma indicate that, when using lasers in the blue–green region of the spectrum, the occurrence of a RR effect must also be carefully considered for other biofluids.

Effects of different substrate–biofluid mixing procedures

When using metal colloids as substrates, SERS spectra may depend on how the colloids are added to the sample, and in particular on the colloid–biofluid volume ratio. SERS spectra usually depend on pH [72–76], which controls the electrostatic interactions between analytes and substrate. Each type of metal colloid has its own pH, and the pH of different biofluids can also vary substantially. For instance: blood, plasma, and serum have a well-defined pH of 7.4 [77], whereas urine has a broad range of possible pH values, from 4.5 to 8.0 [78]. Upon mixing a colloidal substrate with a biofluid the pH of the resulting mixture, and consequently the SERS spectrum, will depend on the pH values of the two components and on the ratio of their relative volumes.

Moreover, the ratio between the total number of nanoparticles and the volume of biofluid will affect how analytes will adsorb on the substrate, and consequently affects the SERS spectrum. The greater the nanoparticle density in a colloid–biofluid mixture, the larger the surface available for molecular

adsorption. When the ratio between the total number of nanoparticles and the volume of biofluid is large, there will be enough surface available to adsorb a varied population of analytes, from those with a high binding affinity (adsorbing rapidly) to those with a low binding affinity (adsorbing slowly). Conversely, when this ratio is small, the surface available for adsorption is small and those analytes with a high binding affinity will adsorb first, possibly saturating the available surface. A consequence of these two effects is that, for the same biofluid, mixtures prepared using different colloid–biofluid volume ratios can lead to different populations of adsorbed species, and hence to different SERS spectra, as recently reported for spectra of serum and plasma [46].

Anomalous bands and artifacts commonly observed in SERS

For several reasons, SERS spectra often have anomalous bands and artifacts, which could be incorrectly assigned to analytes (or, in the case of biofluids, to metabolites) if not properly identified. Such anomalous bands have already been described in literature [27, 79], and we will briefly mention only those which, in our opinion, are the most relevant to SERS of biofluids.

SERS spectra often contain bands caused by reagents used for substrate preparation, or by side products of the reactions involved. For instance, citrate is commonly used as a reagent to prepare metal colloids, and un-reacted citrate ions adsorbed on the nanoparticle surfaces act as electrostatic stabilizers. Thus, citrate bands at approximately 1400, 1020, and 950 cm^{-1} are often observed in SERS spectra obtained using metal colloids [79]. Moreover, citrate is often used as an anticoagulant in plasma, and citrate bands were reported in SERS spectra of plasma when such an anticoagulant was used [46].

Anomalous bands might also be caused by trace contaminants, for example unidentified "Rhodamine-like" species whose bands at 1650, 1575, 1510, 1363, and 1309 cm^{-1} are often observed in SERS spectra obtained using Ag colloids and excitation in the green region of the spectrum [79], and can be easily mistaken for bands resulting from the sample [66]. Such bands were observed in SERS spectra of serum and plasma when using Ag colloids and a 514.5 nm laser [46].

Photodegradation may also affect SERS spectra, especially those collected from non-colloidal substrates, if adequate measures are not taken to avoid it. Graphitic or amorphous carbon, whose formation is often a consequence of photodegradation in SERS experiments, yields intense and broad bands at approximately 1560 and 1360 cm^{-1}, caused by stretching modes of bonds between sp^2 carbons, which might be observed in SERS spectra [27]. Photodegradation is seldom a problem with colloidal substrates, because heat is efficiently dissipated by water and Brownian motion causes a continuous renewal of the probed nanoaggregates. For non-colloidal substrates, reducing the density of laser power (for instance by reducing laser power or by increasing the diameter of the laser spot) or continuously changing the probed surface (e.g. moving the sample with respect to the laser spot) usually helps limit the occurrence of sp^2 carbon bands [27].

Common biofluids: characteristics and SERS spectra

Blood, plasma, and serum

Blood is the biofluid permitting exchanges of substances between different bodily tissues [77]. It comprises a cellular component and a liquid component (plasma). The cellular component (40–50 % of blood volume) consists of three types of cell:

1. erythrocytes, for transport of respiratory gases;
2. leukocytes, acting as a first defense against pathogens; and
3. platelets, responsible for hemostasis (i.e. the arrest of bleeding).

Plasma largely consists of water (91–92 %) and proteins (7 %), plus mineral salts and a variety of organic compounds, including carbohydrates, lipids (transported by proteins), amino acids, nucleotides, and a great number of metabolites (>4600 identified so far [80]). Albumin is the most abundant of the plasma proteins (54 %), but many other proteins are also present: immune-system proteins (globulins), proteins involved in coagulation (e.g. fibrinogen), enzymes, hormones, and many others. Plasma has many functions: from the regulation of hydro-mineral, carbohydrate, lipid, and protein metabolism, to the maintenance of the osmotic pressure. Serum is the liquid component of blood that remains after coagulation, differing from plasma because of the clotting factors, which are only present in the plasma. The pH of plasma and serum for a healthy subject is strictly regulated by a buffering system to be in the range 7.35–7.45.

Because of the difficulties related to the presence of cellular components, only a few papers have reported SERS spectra of whole blood [52, 64, 65], whereas several studies applying SERS to serum and plasma are available [46, 47, 49–51, 55, 81]. Moreover, several studies are available on SERS of isolated erythrocytes (i.e. red blood cells), with no plasma or other cellular components present [70, 71, 82–84]. As previously explained, SERS spectra of whole blood mainly reveal the RR bands of hemoglobin when excited in the blue–green region of the spectrum [52, 65], as in the case of SERS spectra obtained from isolated red blood cells under resonance conditions [70, 82–84]. On the other hand, Premasiri et al. reported that, unlike normal Raman of whole blood, SERS spectra

obtained with an excitation in the near-infrared at 785 nm (i.e. off-resonance with respect to the heme) do not have hemoglobin bands [64]. Interestingly, the spectrum of whole blood reported therein is extremely similar to that of serum, suggesting that the cellular component of blood does not have a substantial effect on the SERS spectrum (Fig. 2). It must be noted, however, that the SERS spectra in that study were obtained from a dried thin blood film deposited on a non-colloidal SERS substrate consisting of aggregated Au nanoparticles immobilized onto a SiO_2 support. Unfortunately, no SERS spectra of whole blood obtained from colloidal substrates and excited in the near-infrared are yet available, so that it is still to be ascertained if the use of metal colloids with blood would yield similar results. However, Drescher et al. reported that SERS spectra of isolated red blood cells, obtained upon incubation with metal colloids and using near-infrared excitation, have several hemoglobin bands [71]. Such bands are possibly caused by hemoglobin molecules released by the cells because of the hemolysis (i.e. the breaking of the erythrocyte membranes, and the consequent release of their contents) induced by the metal nanoparticles [71]. Indeed, hemolysis is known to be a problem upon incubation of blood with metal colloids [71, 85, 86], especially for Ag nanoparticles, and should be carefully considered when collecting SERS of blood using colloidal substrates.

SERS spectra from serum and plasma were reported by several groups, on both Ag and Au colloidal and non-colloidal substrates, using near-infrared excitation (Table 1). Irrespective of the metal used, most reported colloid-based SERS spectra of these two biofluids can be divided in two "types", each type having a characteristic spectral profile (Fig. 3), depending on how the spectra were obtained. One type of spectrum ("type A") is obtained upon addition of plasma or serum to a concentrated metal colloid in a 1:1 volume ratio, or to a non-concentrated colloid in a 1:9 volume ratio [46, 53, 54, 56, 57, 87]. The other type ("type B") is obtained by mixing plasma or serum and non-concentrated colloid in a 1:1 volume ratio [46, 49–51], suggesting that the spectral profile depends on the final concentration of metal nanoparticles, as previously mentioned. Interestingly, SERS spectra of plasma or serum obtained by a near-infrared excitation on solid-supported metal nanoparticles [47, 64] (i.e. non-colloidal substrates) share several characteristics with "type A" spectra obtained from the same biofluids with colloidal substrates (Figs. 2 and 3). Both A and B spectrum types can be obtained from serum and plasma in the absence of clotting factors and serum proteins [46], indicating that these two blood components also do not have an important effect on SERS spectra of these biofluids, and suggesting that SERS bands are mostly associated with low-molecular-weight (i.e. <3 kDa) metabolites. Indeed, many bands of SERS spectra of plasma and serum can be confidently assigned to uric acid and hypoxanthine [46, 64] (Fig. 3), but all the remaining bands are only tentatively assigned on the basis of the Raman shifts available in literature (see for instance assignment tables in

Table 1 Literature overview on label-free SERS of biofluids

Biofluid	Substrate type	Laser (nm)	Substrate metal	Refs.
Whole blood	Colloidal	514	Ag	[52]
	Non-colloidal	532	Ag	[65]
		785	Au	[64]
Plasma	Colloidal	633	Ag	[46]
		785	Au	[46, 53]
			Ag	[46, 54, 56, 57, 87]
	Non-colloidal	785	Au	[64, 88]
Serum	Colloidal	633	Ag	[46]
		785	Au	[46, 55]
			Ag	[46, 49–51, 81]
	Non-colloidal	633	Ag	[89]
		785	Ag	[47]
Urine	Colloidal	633	Au	[60]
		785	Au	[61, 90, 91]
		785	Ag	[59]
	Non-colloidal	633	Au	[92]
		633	Ag	[93]
		785	Ag and/ or Au	[94]
Saliva	Colloidal	633	Ag	[62]
	Non-colloidal	633	Au	[95]
		785	Au	[96–98]
			Ag	[99]
Tears	Colloidal	532	Ag	[63]
	Non-colloidal	633	Au	[92]
Semen	Colloidal	785	Ag	[69]

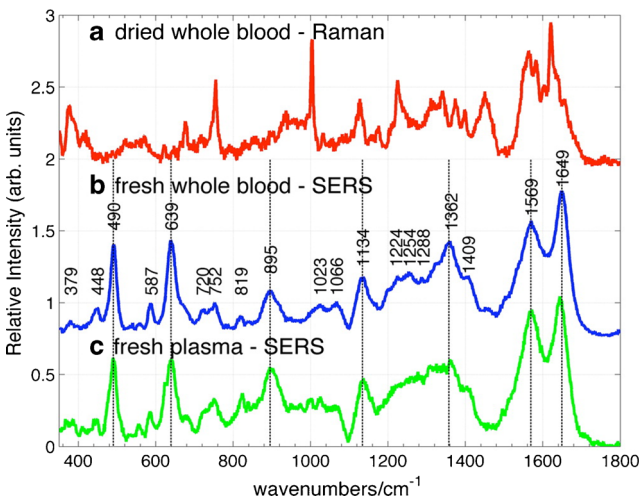

Fig. 2 SERS spectra of (*b*) whole blood and (*c*) fresh plasma, compared with (*a*) normal Raman spectrum of dried whole blood, excited at 785 nm. Reprinted, with permission, from Ref. [64]

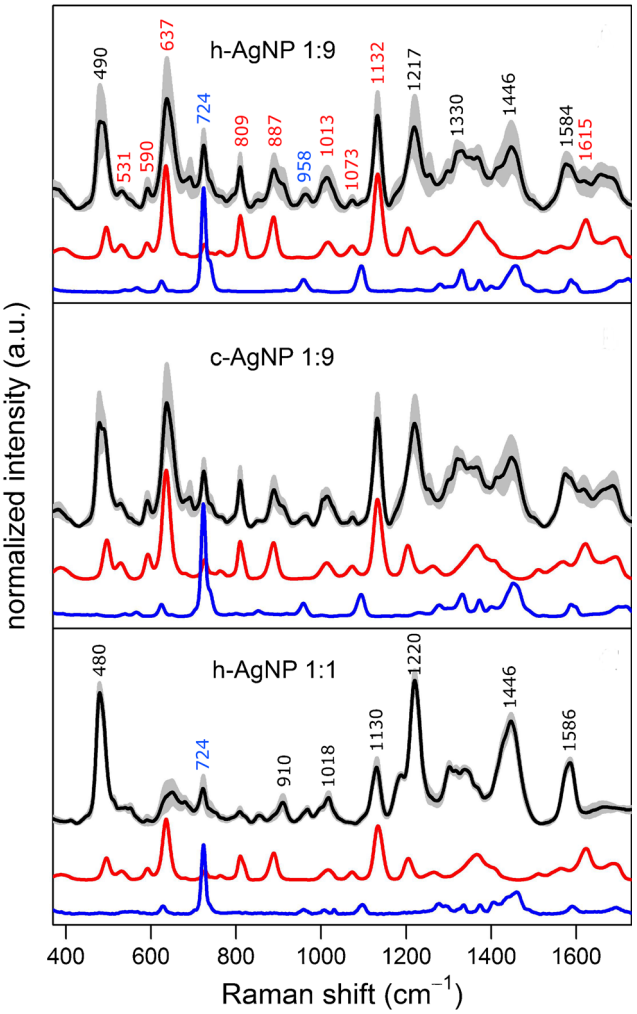

Fig. 3 Average SERS spectra of filtered serum (*black lines*) obtained on different metal colloids and in different serum-to-colloid volume ratios: hydroxylamine-reduced Ag colloid (h-AgNP) in volume ratios of 1:9 (*top*) and 1:1 (*bottom*), and citrate-reduced Ag colloid (c-AgNP) in a volume ratio of 1:9 (*middle*). Spectra obtained from 1:9 volume ratios have similar characteristics ("type A" spectrum), whereas the spectrum obtained from a 1:1 volume ratio is different ("type B" spectrum). Mean spectra were averaged over 20 spectra of different healthy subjects. Intensity standard deviations for serum spectra are shown as *grey* shaded areas. SERS spectra of uric acid (*red lines*) and hypoxanthine (*blue lines*) are shown for comparison. Excitation at 785 nm. Reprinted, with permission, from Ref. [46]

Refs. [50, 53]). Hypoxanthine and uric acid bands are more intense in "type A" spectra, whereas the assignment of the main bands in "type B" spectra is still uncertain.

It should be stressed that band assignment in SERS is not trivial, because a SERS spectrum depends on the adsorption geometry of the analyte (by the so-called "surface selection rules" [100–102]) and can have relative intensities that depend on experimental conditions (e.g. pH, molecular crowding at the surface). For the same reasons, SERS spectra can appear very different from the corresponding normal Raman spectrum, so that the normal Raman spectra found in literature are seldom useful for band assignment.

Despite the difficulties involved, a reliable assignment of all the bands observed in SERS spectra of blood, serum, and plasma upon near-infrared excitation would be of primary importance for the development of diagnostic applications, enabling a biochemical insight into SERS spectra of these biofluids which is now absent.

Urine

Urine is a waste biofluid formed as a consequence of the elimination of terminal products of metabolism and exogenous substances that require clearance from the bloodstream [77, 78]. A healthy adult produces 600–1600 mL urine per day, with a pH in the range 4.5–8, depending on the time of the day and on the diet. The concentration and osmolarity of the urine can vary substantially, and are closely correlated with the state of hydration of the body and with health conditions. The protein content depends on specific pathological or physiological conditions (e.g. intense physical activity), but in healthy adults it is usually low (<10 mg dL^{-1}), with albumin being one of the major components (40 %). However, urine is rich in metabolic information, containing more than 2600 different metabolites [103]. The urinary sediment (i.e. a usually low amount of solid matter, settling to the bottom of a sample that has been allowed to stand for several hours) is composed of cells (epithelial cells and red and white blood cells), but bacteria and crystals may be present as well. Most of the crystals (e.g. uric acid, oxalates, carbonates, and phosphates) are formed after urination (as a result of pH or temperature variations), but they are also often observed in pathological conditions. Urine is readily available and easily collected by non-invasive procedures, and thus is of great interest for diagnosis and screening applications.

Several groups have reported SERS spectra of urine (Table 1) obtained using a variety of substrates and excitation wavelengths. In contrast with serum and plasma, whose SERS spectra in literature have similar characteristics, urine spectra reported by different groups are diverse, so that defining one or two SERS spectra types with definite and constant characteristics is not possible. Some spectra clearly have intense bands associated with creatinine [60, 90], and some are dominated by an intense band at approximately 1000 cm^{-1} associated with urea [59, 91–94]. In another study, the most intense band is at 721 cm^{-1}, and is attributed to hypoxanthine [61]. Such diversity could possibly be explained by the different substrates and excitation wavelengths used for each experiment, because experimental conditions are known to strongly affect SERS spectra. Another reason for the differences observed could be the extremely high inter and intra-individual variability of urine in terms of composition, concentration, and pH. The lack of a systematic pattern in the data reported so far prevents any general conclusion about the interpretation of the SERS spectra of urine.

Saliva

Saliva is a limpid aqueous solution produced by the salivary glands, mainly acting as a detergent of the teeth and the oral cavity and as a lubricant for food ingestion [77]. The daily volume produced by a healthy adult is approximately 1–1.5 L day^{-1} (pH 6.8–7.2). It is mostly constituted by water (99.5 %), and also contains a variety of solutes including inorganic ions (e.g. K^+, Na^+, Ca^{2+}, Mg^{2+}, Cl^-, HCO_3^-, $H_2PO_4^-$, HPO_4^{2-}), proteins (immunoglobulins, enzymes), mucus, urea, and uric acid.

As in the case of urine, several groups have reported SERS spectra of saliva (Table 1) which are very different from each other. The variety of experimental conditions used, together with the inter and intra-individual variability of this biofluid, can account for the differences between the reported spectra. Depending on the study, tentative assignments suggest the occurrence of bands resulting from proteins and amino acids [62, 95–97], lipids [62, 95], and DNA and/or RNA bases [62]. Because of this inhomogeneity in the data reported so far, no general conclusions about the interpretation of SERS spectra of saliva can be drawn, and systematic studies on this topic are needed.

Other biofluids

SERS spectra have also been reported for other biofluids, including tears [63, 92] and seminal plasma [69] (Table 1), the latter obtained using polarized laser light. Interestingly, the SERS spectra of these two biofluids share several bands, and are similar to those observed from serum and plasma. In particular, the SERS spectrum of tears [63] has bands resulting from uric acid and hypoxanthine (Fig. 4), similar to those of "type A" spectra of plasma and serum (Fig. 3). SERS spectra of seminal plasma [69], on the other hand, are analogous to "type B" spectra of plasma and serum, with the difference that for seminal plasma an additional intense band at 720 cm^{-1}, possibly associated with hypoxanthine, is present. As in the case of serum and plasma, for the SERS spectrum of seminal plasma only a tentative band assignment is available, based on literature [69].

Diagnostic applications

Biofluids are samples extremely rich in information. A label-free SERS spectrum only contains a fraction of the total information available from a biofluid, because only a fraction of the biofluid constituents adsorb on the metal substrate and contribute to the spectrum. Nevertheless, such partial information can be used for diagnostic purposes, and several diagnostic studies are available, based on label-free SERS of biofluids (Table 2).

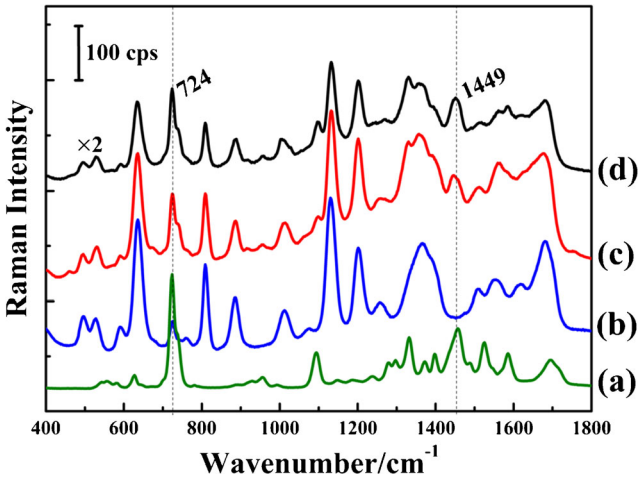

Fig. 4 SERS spectra of tears (*d*) together with hypoxanthine (*a*), uric acid (*b*), and blood serum (*c*), obtained from citrate-reduced Ag colloids. Spectra from tears and serum were collected by mixing concentrated colloids with biofluid in a 1:1 volume ratio. The spectrum of tears was multiplied by a factor of two for better viewing. Excitation at 532 nm. Reprinted, with permission, from Ref. [63]

With the exceptions of a few studies on diabetes [81, 93] and herpes simplex conjunctivitis [104], most diagnostic applications of label-free SERS of biofluids are focused on cancer. SERS-based diagnosis has been performed for nine different types of cancer, using plasma, serum, urine, and saliva (Table 2). Typically, SERS spectra are collected from a group of patients diagnosed with a specific cancer and from a control group (i.e. healthy subjects); this is the case for the data reported in Fig. 5. An exception is a recent study comparing

Table 2 Diagnostic applications of label-free SERS of biofluids

Pathology	Biofluid	Refs.
Oral cancer	Saliva	[95]
Nasopharyngeal cancer	Plasma	[53, 57]
	Serum	[50]
Esophageal cancer	Plasma	[87]
	Serum	[50, 51]
	Urine	[59]
Gastric cancer	Plasma	[56]
	Serum	[50, 89]
Colorectal cancer	Serum	[50, 55, 89]
Liver cancer	Serum	[50]
Lung cancer	Urine	[94]
	Saliva	[62]
Prostate cancer	Serum	[49]
	Urine	[61]
Cervical cancer	Plasma	[54]
Diabetes mellitus	Serum	[81]
	Urine	[93]
AIDS (HIV)	Saliva	[98]

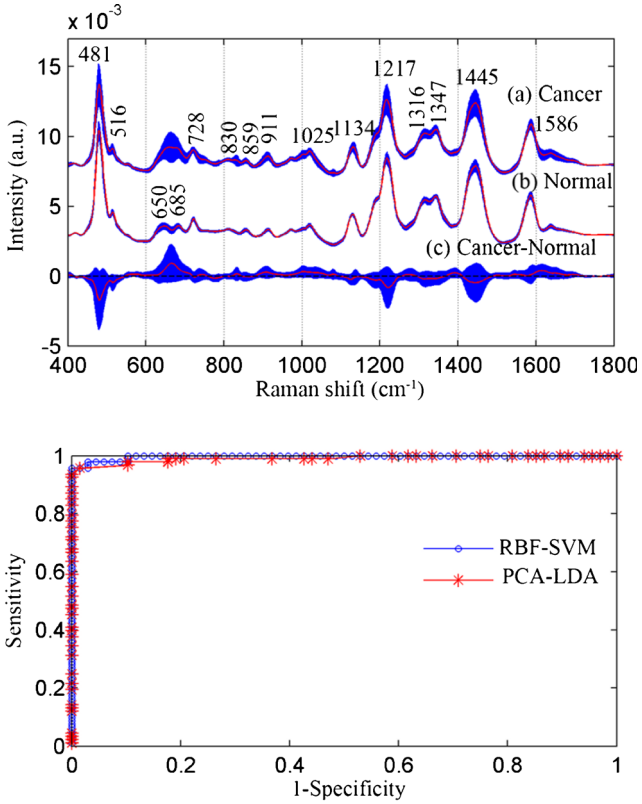

Fig. 5 (*top*) Normalized mean SERS spectra of serum samples, excited at 785 nm, from prostate-cancer patients and healthy subjects, together with their difference spectrum. Spectra were obtained by mixing Ag colloids with serum in a 1:1 volume ratio, incubating for 1 h at room temperature, and letting dry. Standard deviations are represented as *shaded areas*. (*bottom*) Receiver operator curve (ROC) for two different predictive models. Reprinted, with permission, from Ref. [49]

nasopharyngeal cancer patients at two different tumor stages with the control group (Fig. 6) [53]. In most studies [53–57, 59, 61, 62], baseline-subtracted and normalized spectra are transformed into a set of principal components using principal component analysis (PCA) [105, 106] to reduce the number of variables. Then the principal components containing the most relevant spectral information are used to build predictive classification models using linear discriminant analysis (LDA) [105, 106]. In some recent papers [49–51, 87], support vector machine (SVM) algorithms [105, 106] are used instead of LDA for building predictive models. Evaluation of the diagnostic performance of the models is obtained by applying validation methods (e.g. leave-one-out cross validation [105, 106]) and calculating parameters including sensitivity, specificity, and overall accuracy as well as the receiver operator curve (ROC, see Fig. 5 for an example). The values of accuracy, sensitivity, and specificity obtained are usually well above 80–90 %, supporting the hypotheses about the effectiveness of SERS-based diagnostics.

For each biofluid, the average spectral differences reported between patients and control groups are usually specific to each different type of cancer, although, interestingly, a common pattern can be observed as well, irrespective of the cancer form. In particular, with respect to healthy controls, serum and plasma SERS spectra of cancer patients seem to have more intense bands at approximately 720–730 cm^{-1} and 950–960 cm^{-1}, corresponding to hypoxanthine bands [53–57, 61, 87]. Moreover, a band in the 480–490 cm^{-1} region, whose assignment is still uncertain, seems to be less intense for cancer patients than for controls [49, 53–57, 61, 87]. As already stated above, a more complete and reliable band assignment, especially for SERS spectra of serum and plasma, is highly desirable to correlate the observed spectral changes with specific biochemical compounds, possibly helping to identify biomarkers.

The number of subjects included in the analysis varies from study to study, being in the range of 20–90 subjects per group. A recent work by Beleites et al. [107] discussed the

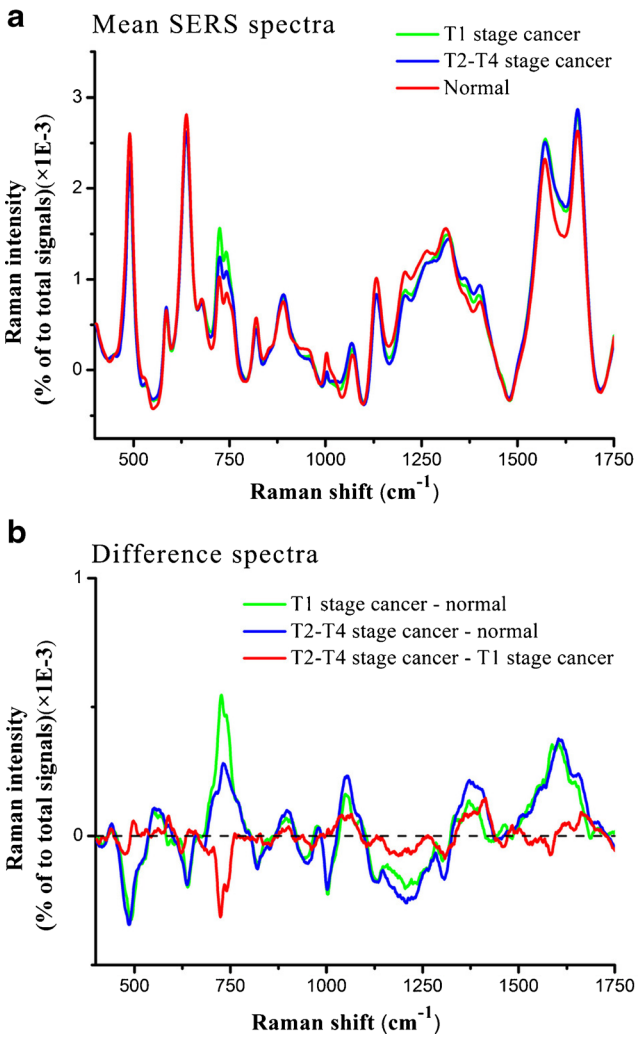

Fig. 6 (**a**) Comparison of normalized mean SERS spectra of blood plasma from healthy subjects and nasopharyngeal cancer patients at different stages (T1 and T2–T4), and (**b**) the difference spectra. Spectra were obtained by mixing concentrated Au colloids with plasma samples in a 1:1 volume ratio, and using a 785 nm excitation. Reprinted, with permission, from Ref. [53]

importance of planning an adequate sample size for getting statistically relevant results out of prediction models obtained from vibrational spectra. According to that paper, 75 to 100 subjects for each class are necessary to achieve a reasonable precision for the values of accuracy, sensitivity, and specificity obtained from validation. However, with the exception of two studies [49, 50], SERS-based diagnostic applications reported so far relied on smaller numbers of subjects, so that the promising preliminary results should be confirmed with larger populations.

It should be noted that, with two exceptions [61, 89], all the studies reported in this review either used samples from patients under chemotherapy or did not give any details on this aspect, leaving it undetermined. Although one study provided preliminary data suggesting that chemotherapy did not affect SERS spectra of plasma [57], an overall effect of chemo or radiotherapy or other kinds of therapy on SERS spectra of biofluids cannot be completely ruled out, and further studies are required to clarify this important aspect.

Conclusions and perspectives

Label-free SERS of biofluids holds great potential for diagnostics, as suggested by the high accuracies, sensitivities, and specificities reported so far for diagnosis of cancer, but many fundamental aspects of this technique still need to be investigated and confirmed. Despite the numerous works reporting applications, very few papers have reported systematic studies on SERS of biofluids. Optimal experimental conditions to obtain intense, reproducible SERS spectra still need to be firmly established for many biofluids, and reliable and consistent band assignments are required. Moreover, given the chemical complexity of biofluids and their inter and intra-individual variability, studies need to be extended as far as possible, increasing the number of subjects involved. However, once these aspects are settled and a firm basis is established, label-free SERS of biofluid could rapidly attain the deserved interest from the medical community.

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