Nanostrucured substrates for surface-enhanced Raman scattering spectroscopy

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This chapter is not meant to be an exhaustive review of the rich variety of metal nanostructures to be used as surface-enhanced Raman scattering (SERS) substrates. The number and diversity of such substrates are so great that such a task would be formidable. For broader and more comprehensive overviews on SERS substrates, the reader is referred to recent reviews [1−3] or books on SERS [4,5].

The purpose of this chapter is rather to introduce the nonspecialist reader to the most important aspects of metal nanostructures when used as SERS substrates to investigate in vitro and in vivo biological samples (e.g., biofluids, cells, or tissues) for diagnostic or theranostic purposes. This chapter purposely targets researchers without direct SERS expertise, addressing a broad audience with a variety of different backgrounds. Thus theoretical aspects, equations, and in-depth technical details will be avoided (the interested reader will be redirected to proper sources), in favor of qualitative explanations.

The chapter consists of three parts: in the first part, the reader is given a very short introduction to basic aspects of SERS, whereas in the second part general aspects of SERS substrates are discussed. The third part specifically addresses aspects relevant to biomedical and diagnostic applications.

6.1 An introduction to surface-enhanced Raman scattering

6.1.1 A brief qualitative description of the surface-enhanced Raman scattering effect

Raman spectroscopy is an optical spectroscopic technique based on the homonymous Raman effect [6–8]: the in elastic scattering of monochromatic light due to molecular vibrations. The effect was theoretically predicted before their experimental discovery [9], which was achieved by Raman and Krishnan in 1928 [10]. In the decades after his discovery, all the different theoretical aspects of the effect were thoroughly described [11]. Leaving aside the rigorous but complex theoretical treatment, the Raman effect can be qualitatively described in very simple terms. When matter is illuminated with a monochromatic light such as that of a laser beam, most of the scattered light retains the same wavelength as the incident light (i.e., elastic or Rayleigh scattering). However, a very tiny fraction of the scattered light coming out of the sample has different wavelengths because of its interaction with the matter (i.e., Raman scattering). In other words, the light–matter interaction "shifts" the wavelength of the incoming light to different extents (the units of the "x axis" of Raman spectra are called "Raman shifts"), so that polychromatic light is generated as a consequence of the energy exchanged between monochromatic light and matter during the scattering process. These wavelength shifts are modulated by molecular vibrations, so that a Raman spectrum is, a vibrational spectrum, where each band corresponds to a specific type (a "normal mode") of vibration. The physical process behind the Raman effect, however, is very different from the absorption process occurring in IR, and despite both spectroscopies are originating from molecular vibrations, IR and Raman spectra are different.

What matters most is that different molecules will vibrate differently: each molecular structure will have its set of "normal modes" of vibrations. A direct consequence will be that a vibrational spectrum will be unique for a specific structure, which is the reason why vibrational spectra are sometimes referred to as "molecular fingerprints." The specificity of Raman spectroscopy is of course a distinct advantage in chemical analysis, and this technique, especially with the more compact, performing, and accessible instrumentation available was proposed as a solution for many analytical applications. In spite of its many

advantages, however, Raman spectroscopy has an intrinsic disadvantage: the Raman effect is a weak effect, and thus the technique is not very sensitive.

SERS offers a solution to overcome this drawback of the Raman effect by enhancing its intensity in the presence of metal nanostructures (Fig. 6.1). SERS was serendipitously discovered in the 1970s while studying the behavior of pyridine adsorbed on Ag electrodes [12,13]. After a careful consideration of all the experimental aspects, researchers concluded that the only possible explanation for the spectra observed was that the intensity of the Raman bands due to the pyridine adsorbed on the roughened Ag electrodes was enhanced by a factor of 10^6 with respect to the normal Raman spectrum observed from pyridine in a bulk solution. Two very important aspects of SERS that define this technique have been evident since and should be stressed right from the start: (1) SERS can boost Raman sensitivity: Raman spectra from very low amounts of substance can in fact be observed exploiting SERS, overcoming the intrinsic problem of the poor sensitivity of Raman spectroscopy; (2) SERS is a surface technique: enhanced Raman spectra can be observed only from species adsorbed on (or very close to) a metal surface, so that phenomena occurring at the metal–solution interface (e.g., catalysis) can be studied.

For a long time, the origin and explanation of the SERS effect was debated and, in part, it still is [14]. Experimental findings showed that both the morphology and the chemical nature of the metal surface played a central role, since flat surfaces did not show a significant enhancement and different metals lead to various results (with Ag, Au, and Cu being the most effective), but how and why remained a matter of debate. In time, the SERS community slowly built a consensus toward an explanation

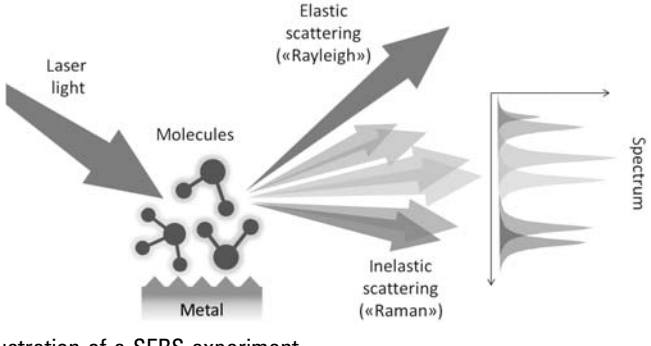

Figure 6.1 Schematic illustration of a SERS experiment.

involving two main mechanisms of enhancement: the so-called electromagnetic mechanism (EM) and the chemical mechanism (CM), which could explain the experimental results observed. Details about these mechanisms are not reported here: excellent books address this aspect in detail [15,16]. It is now generally accepted that the EM is accountable for most of the SERS effect, and that this effect has to do with the presence of localized surface plasmons (i.e., collective oscillation of surface electrons) in metal nanostructures. This description well accommodates the fact that coinage metals such as Ag, Au, and Cu display a SERS effect with a laser excitation in the visible or near-IR range and that the enhancement effect rapidly decays with the distance from the metal surface. This theory also explains why specific surface morphologies involving nanostructures are needed, since plasmonic properties adequate for the SERS effect when using visible or near-IR excitation only arise from metal nanostructures. Today, our grasp of plasmonics allows us to design metal nanostructures tailored to have localized surface plasmons at specific wavelengths, which can be then realized via nanofabrication techniques. Experimental results confirmed theoretical predictions, so that EM is a well-understood and consolidated SERS mechanism.

6.1.2 Experimental aspects

SERS is not an easy, straightforward technique to use. There are many experimental aspects to take into account when planning a SERS experiment, and an experimenter with some experience in SERS will be able to determine the best conditions to maximize the chances of success (i.e., to observe an intense SERS spectrum from a specific analyte).

6.1.2.1 Matching substrates and laser wavelengths

The first and perhaps most important aspect to take into account is the fact that not all substrates will work with all analytes and with all lasers [17]. The EM theory tells us that, for instance, given a metal surface, the choice of the exciting laser is limited. In the best case, one can design and realize a substrate having exactly the desired characteristics to maximize the match between the surface plasmons frequency and that of the exciting laser, but most often one has to work with a given substrate, or has a limited choice of excitation lasers, and has to get the most out of it. As a good rule of thumb, Ag substrates work well with a broad range of excitation sources: Ag nanoparticles,

for instance, display a SERS effect when excited with lasers having wavelengths in the blue/green region (e.g., using 514/532 nm lasers, but also with 405/413 nm), but yield intense SERS spectra also upon near infrared excitation (e.g., 785 nm) [17]. Au substrates, on the other hand, depending on their characteristics, may work well upon red (e.g., 633 nm) or near-IR (e.g., 785/830 nm) excitation.

6.1.2.2 Chemical nature of the analyte

Of utmost importance is also that the analyte should be close to the nanostructured metal surface. Ideally, it should be in direct contact with the metal surface (i.e., physisorbed or chemisorbed). An experienced SERS spectroscopist will predict if such interaction have good chances to occur just by looking at the molecular structure of the analyte. Thiols, amines, N-containing heterocyclic compounds, and carboxylic acids do have a strong interaction with Ag and Au substrates, and thus they are expected to yield intense SERS spectra.

Sometimes a direct interaction is difficult or impossible. Nonpolar molecules lacking the functional groups listed above or carbohydrates, for instance, will not have a strong interaction with Ag or Au surfaces, and SERS spectra of those kind of molecules are notoriously difficult to get, unless chemical (e.g., chemical bonding to an already adsorbed species) or physical methods (e.g., electrostatic interaction) are used to "attract" these analytes close to the surface.

Special attention should be paid to electrostatic charges. Depending on the conditions (e.g., pH of the environment, adsorbates already present on the substrate) the metal surface and the analyte might have definite electrostatic charge. For instance, citrate-reduced Au and Ag nanoparticles, a kind of very common and widely used SERS substrates, have citrate molecules with negatively charged carboxylate groups adsorbed on the metal surface, conferring an overall negative charge to the surface. With such substrates it will be very difficult to get a SERS signal out of analytes having a definite negative charge. This is one of the reasons why in SERS experiments pH should be carefully controlled and checked, since a slight change in the pH might dramatically influence the analyte−substrate interaction, and thus the SERS spectrum generated.

In general, in view of the subtle dependence on the specific analyte−metal interaction, of the influence of the environmental conditions, and on the wavelength-metal matching, it can be stated that no such thing as a "universal" SERS substrate exists

that can be generally used with all possible analytes. Each analyte, or even better, each analytical problem, requires a specific SERS solution, which is one of the reasons SERS is a tricky technique to be used by nonexperts.

6.1.2.3 Environmental conditions

In general, environmental conditions such as pH and ionic strength (i.e., concentration of charged species in solution) can have a dramatic impact on a SERS experiment, not only for the analyte−substrate interaction but also for the substrate itself. In the case of Au and Ag colloids, for instance, the formation of nanoparticle aggregates is functional to the generation of adequate plasmonic nanostructures [18,19]. Such aggregates can be formed by increasing the ionic strength of the environment, which will shield the surface charges responsible for the colloid stability, leading to nanoparticle aggregation. Sometimes the ionic strength is increased by the addition of salts to the colloid, but other times the analyte solution already has the necessary ionic strength to lead to nanoparticle aggregation. Working with analytes in buffered saline solutions rather than in water is often desirable in SERS experiments using colloidal Ag or Au as substrates, since pH is well defined and the ionic strength is often already high enough to induce nanoparticle aggregation.

6.1.2.4 Interference from other species

Often, more than one species is present together with the analyte in the solution: these can be buffering species, salts, or other molecules, as in the case of "real," chemical complex samples such as biofluids. In these cases, the problem of competing species might arise. Species other than the analyte might compete with the analyte itself for the metal surface, so that the SERS spectrum observed contains intense bands that are not due to the analyte, but to the competing species. Sometimes, the competing species are already present on the substrate, as impurities or as a "capping" agent, which is intrinsically part of the substrate because of the synthetic method used for its preparation (e.g., the citrate ions present on the surface of the citrate-reduced metal colloids). Thus observing strong SERS bands in a SERS experiment does not necessarily mean that the bands observed are due to the analyte [20]. When using metal colloids as substrates, a common mistake is to interpret the intense bands due to citrate as those of the analyte. A direct comparison between the normal Raman spectrum of the analyte and the SERS spectrum obtained must always be done to

ensure that the signal observed is actually due to the analyte, and not to interfering species. Impurities can also originate spurious SERS bands, as well as amorphous carbon that might form as a consequence of sample photodegradation [20].

6.1.2.5 Photo-induced thermal degradation

Thermal degradation of adsorbed species as a consequence of intense illumination is, in fact, another common problem for SERS, which often leads to an intense background due to two very broad bands around 1300 and 1600 cm^{-1}, a distinct marker for the presence of amorphous carbon. In SERS experiments, a good practice is to look for these bands and, in case, to decrease the laser power until no bands to amorphous carbon are detected.

6.1.2.6 Normal Raman and fluorescence: competing processes

Raman and fluorescence are two processes that are also competing with SERS. Fluorescence is often responsible for intense, sloping backgrounds underlying SERS bands. In worst cases, fluorescence (from the analyte or from impurities) can completely submerge the SERS bands, and no SERS spectrum is observed. Usually, however, excitation in the near-IR will minimize the interference from fluorescence, making the observation of SERS spectra possible from otherwise fluorescent samples. Sometimes, normal Raman bands from solvents or concentrated interfering species will also contribute to the spectrum. For instance, analyte solutions containing fractions of solvents such as methanol, ethanol or DMSO, often used to prepare solutions of poorly soluble analytes, might display normal Raman bands of these substances beside the SERS bands of the adsorbed analyte. This is one more reason why it is always advisable to check a spectrum of the "blank" sample (a solution with no analyte present) to get an idea of which bands are due to the matrix itself rather than to the analyte.

6.1.3 Surface-enhanced resonance Raman spectroscopy: resonance effects

Many analytes are "colored," that is, they present electronic transitions in the visible or near-IR region, such as the pi−pi or n−pi transitions for organic molecules having extended systems of conjugated double bonds. When such analytes are probed with a laser with a wavelength corresponding to an energy similar to one of their electronic transitions, absorption processes

occur, and the probability associated with the transitions involved in the Raman process is greatly enhanced. In that case, the intensity of the Raman bands is enhanced, and the overall resonant Raman effect is exploited as an enhancement mechanism in what is called “resonance Raman spectroscopy” or RR spectroscopy [7]. This effect can take place even when resonant analytes are adsorbed on nanostructured metal surfaces in the course of a SERS experiment, yielding very intense spectra that benefit from the synergistic combination of both SERS and RR effects. When this is the case, the term used is “double R” SERRS [i.e., surface-enhanced resonance Raman spectroscopy (SERRS)] [7,21]. Because of this RR effect, one always has to keep in mind that the choice of the excitation wavelength, and consequently of the nature of the metal substrate, has to take into account resonant transitions. In other words, if your analyte is “colored,” different excitation wavelengths can lead to very different results (i.e., SERS or SERRS), depending on if the RR effect is present or not. Usually, the combination of SERS and RR effects in SERRS yield spectra so intense (detection of single molecules SERRS spectra have been repeatedly and consistently reported) that this is purposely exploited to boost the sensitivity of the method. Sometimes, however, SERRS bands due to resonant impurities present in the sample might interfere with the detection of the analyte bands. In any case, one has to remember that a wanted or unwanted RR effect might greatly affect SERS experiment, and thus the choice of a proper excitation wavelength is of utmost importance.

6.1.4 Enhancement factors

The term EF, in the context of SERS, is a multifaceted and often misunderstood (and misused) word. Several different definitions of EF have been proposed, creating some confusion [22]. In fact, it is a concept created to quantify with a number how much the signal observed in a SERS experiment is enhanced with respect to a normal Raman experiment. Often, this number is meant to quantify “how good” a SERS substrate is, compared to other substrates. However, things are complicated by the fact that the intensity of the SERS signal also depends on the analyte and on the laser used (to name the two most important factors), so that the EF cannot refer to the substrate itself, but to the substrate−laser−analyte combination used.

A general definition of the EF, which assumes that two experiments (i.e., a SERS one and a normal Raman one) are

performed with the same analyte, is

$$EF = \frac{I_{SERS}/N_{Surf}}{I_{RS}/N_{Vol}}$$

where I_{SERS} is the intensity of the SERS signal and N_{Surf} is the number of molecules adsorbed on the metal surface of the SERS substrate in the SERS experiment; I_{RS} is the intensity of the normal Raman signal and N_{Vol} is the average number of molecules in the scattering volume for the normal Raman experiment [22]. This general definition, however, presents some difficulties. While the term N_{vol} can be calculated as the product of the molar concentration for the volume probed by the laser and whose signal is collected by the collection optics, the term N_{Surf} is much more difficult to estimate, as it depends on the affinity of the analyte for the surface. Moreover, this definition assumes that all the molecules adsorbed on the surfaces are equally contributing to the SERS signal, which is not true in general.

A much more viable definition is that of the analytical enhancement factor (AEF), as

$$AEF = \frac{I_{SERS}/c_{SERS}}{I_{RS}/c_{RS}}$$

where c_{SERS} and c_{RS} are the analytical molar concentrations of the analyte in the SERS and normal Raman experiments, respectively [22]. The AEF can be readily calculated, enabling a comparison between different substrates, if the same analyte is used. However, it should be stressed that, since the SERS signal depends on how a specific analyte is interacting with the surface, the results obtained with one analyte might not hold true for others. In other words, while one substrate could be better than another in enhancing the signal of an analyte, the reverse could be true when a different analyte is used. Thus the information given by EFs should be used with care: EFs are useful to compare the performance of different substrates on the same analyte, but extending their use farther than that might be dangerous.

6.2 Surface-enhanced Raman spectroscopy substrates: classification and general characteristics

The availability of nanostructured metal substrates with adequate plasmonic properties is central to SERS. Since the beginnings of SERS on electrochemically or chemically

roughened electrodes, many other substrates have been proposed and used. Metal colloids were one of the first substrates to be used besides roughened electrodes, and rapidly became popular because of their ease of preparation and use, and they are still commonly used today. With the development of nanofabrication techniques and of wet nanotechnology synthetic protocols, a broad variety of SERS substrates have been prepared, so that the literature about this topic is evergrowing, and in recent years many commercial substrates became available as well. Given the wide variety of approaches and characteristics, there are many ways in which SERS substrates can be categorized, besides the obvious criterion of the nature of the metal itself.

A very general criterion is to roughly divide the substrates into colloidal and noncolloidal, where the first are constituted by all those substrates made of metal nanoparticles dispersed into a liquid medium, forming a colloid. A problem with this criterion is that the class of noncolloidal substrates (sometimes referred to as "solid substrates") is very heterogeneous.

Another general criterion would be about the nature of the synthetic method used to prepare the substrates: chemical methods (e.g., chemical etching, electrochemical roughening, wet synthesis of nanoparticles by reduction of metallic salts, etc.) or physical methods (e.g., metal sputtering, electron beam nanolithography, nanoimprinting, laser ablation, etc.). However, in many cases a combination of the two approaches is used, so that this criterion is not very efficient.

A third general criterion would be, from the perspective of nanotechnology, the "direction" of the substrate preparation: top-down or bottom-up. Bottom-up substrates would be the ones prepared using already available building blocks are assembled as elements to form the final nanostructure. An example of bottom-up SERS substrate would be a solid substrate whose surface is constituted by self-assembled metal nanoparticles. Top-down substrates, on the other hand, are the ones prepared starting from a bulk material and forming the nanostructure by "sculpting" it, taking away the parts in excess or shaping it so that what is left in the end is the desired nanostructure. An example of a top-down SERS substrate would be a nanostructured surface obtained by selectively etching parts of the original surface thanks to nanolithographic processes.

A fourth criterion, applicable to noncolloidal substrates, is concerning the "regularity" of the metal nanostructures. Such surface structures can be regularly spaced and ordered, such a

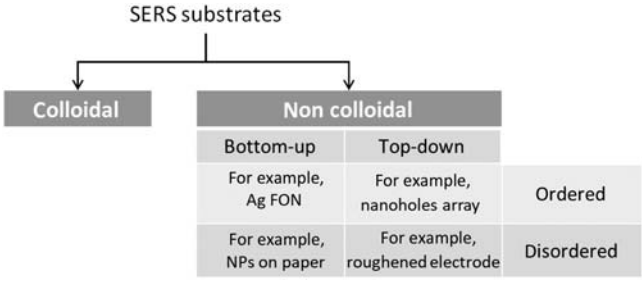

Figure 6.2 General classification of SERS substrates.

regular array of nanoholes or nanodomes, or they can be randomly spaces, irregular and disordered.

None of the criteria above, or any other possible criterion (e.g., the chemical or physical characteristics of the metal surface), is generally accepted as a "universal" criterion capable of categorizing completely and unmistakably the vast universe of proposed SERS substrates. However, with respect to biomedical applications in particular, where samples are often biofluids, cells, or tissues, it might be useful to combine two of the above criteria, first roughly dividing the substrates into colloidal and noncolloidal and then further subclassifying the noncolloidal substrates as bottom-up or top-down (Fig. 6.2). This categorization is proposed for practical purposes (i.e., keeping in mind applications). In fact, samples such as biofluids or cells, for instance, behave very differently when put together with colloidal or noncolloidal substrates for SERS analysis, so it makes sense to use this criterion when describing the use of different types of SERS substrates.

6.2.1 Colloidal substrates

Because of their simple and straightforward synthetic protocols, low costs of reagents needed, the use of basic, inexpensive laboratory equipment, and most important, their effectiveness in enhancing the Raman signal, metal colloids have been and still are widely used as SERS substrates. Although metal colloids made of various transition metals have been reported as SERS substrates, the most commonly used metal colloids are those made of Ag and Au. Different preparation protocols lead to differences in shape and size of the nanoparticles obtained, with a broad variety of morphologies (e.g., nanospheres, nanostars, nanocubes, nanorods, nanoflakes, nanohollow spheres, etc.)

and sizes, from tens to hundreds of nanometers. The shape and size of the nanoparticles, besides the nature of the metal, define the plasmonic characteristics, so that surface plasmons can be tuned to match the exciting laser to be used. For instance, for spherical nanoparticles, the larger the size, the smaller the frequency of the surface plasmons [23,24]. The size of nanoparticles is usually a factor to take into account when planning a SERS experiment. The concept of size is easy to apply only when considering spherical nanoparticles or nanoparticles having a regular, symmetric shape (e.g., nanocubes, nanooctaedra, etc.), whereas the description of more complex shapes such as nanorods or nanoplates require the specification of different sizes along different nanoparticle axes. The general concept is that a minimum size is necessary to generate a significant SERS effect, so that nanoparticles with a size of few nanometers will not show any enhancement [25−27]. For spherical Au nanoparticles, a SERS effect is reported only when using nanoparticles of at least 30−40 nm in diameter [26]. For Ag nanoparticles, the correlation is less well defined, but the fact that the SERS signal depends on the nanoparticle size is established [27].

Anisotropic shapes often have more than one plasmonic frequency, such as nanorods [28] or nanostars [29,30]. The literature is richer in protocols for the preparation of Au colloids than for Ag colloids, and the shape and size of Au nanoparticles can be better controlled than that of Ag nanoparticles, for which the choice is still somewhat limited. In spite of this wide range of choices, however, for most applications just few of these recipes (or their variants) are used. For most applications involving the direct detection of analytes in aqueous solutions, quasispherical nanoparticles dispersed in an aqueous medium are mostly used.

Ag and Au nanoparticles obtained by reduction of metal ions in $AgNO_3$ or $HAuCl_4$ (or $AuCl_4^-$) with citrate ions (in brief: citrate-reduced Ag and Au colloids) were among the first SERS substrates to be used, and are still widely used [31,32]. They have the advantage of being simple one-step synthetic protocols in aqueous environment, carried under mild experimental conditions using a readily available apparatus. Moreover, the obtained colloids are rather stable, if kept in the dark at room temperature, and can be stored for months without losing their function as SERS substrate. The colloidal stability is due to the layer of adsorbed citrate ions, conferring a negative charge (a zeta-potential below −30 mV) to the nanoparticle surface that hinders aggregation thanks to the interparticle electrostatic repulsion. With the citrate-reduction method, it is possible to

obtain spheroidal Au nanoparticles having well-defined sizes [26]. A strong correlation between particle size and the maximum of the extinction band was reported, so that an indication of the size can be simply obtained from an extinction spectrum. A rather different situation is encountered in the case of citrate-reduced Ag nanoparticles [32], for which nanoparticles with a broad range of shapes (mainly spheroids, rods, plates) and sizes are obtained.

Another widely used protocol for obtaining Ag colloids is using hydroxylamine hydrochloride as reducing agent [33], leading to spherical Ag nanoparticles of sizes ranging from 23 to 67 nm, depending on the ratio between the reagents. In that case the surface of the nanoparticles is also negatively charged, but because of the presence of adsorbed chloride ions. Limiting the adsorbates on the nanoparticle surface to simple mono-atomic ions promotes the adsorption of other analytes, which do not need to displace adsorbed molecular species, such as citrate ions or other capping agents. Metal colloids prepared by laser ablation [34] also present the advantage of having a "naked" surface, devoid of molecular adsorbates.

In general, different synthetic protocols lead to nanoparticle surfaces with different physical and chemical characteristics. Often, the production of nanoparticles with more complex shapes requires the use of selective capping agents binding onto specific crystal facets to control the direction of crystal growth, and such capping agents need to be used in organic solvents and are difficult to remove, hindering the adsorption of the analyte on the metal surface, and interfering with the SERS analysis. Problems related to the use of capping agents binding too strongly to the metal and to the need of organic solvents (usually interfering because of their own intense Raman spectrum) as dispersing medium are limiting the use of many metal colloids other than the simple, quasispherical metallic nanoparticles dispersed in aqueous media.

In spite of the wide choice of colloidal syntheses available, no protocol or shape is accepted as "standard," and in the absence of standards each lab use its own recipe. This lack of standardization, together with the well-known repeatability issues linked to colloidal synthesis, makes a direct comparison of results obtained by different labs problematic, and constitutes a serious obstacle to the development of SERS as a standard analytical technique to be used outside academia. Moreover, the most efficient plasmonic nanostructures obtained from metal colloids are the nanoparticle aggregates, which help the formation of nanosized gaps between particles (called hotspots [35]) where the electromagnetic field amplification as required by the EM is particularly

intense. Although still debated, evidence supports the fact that the SERS effect from single spherical metal nanoparticles is negligible with respect to that of aggregates [18]. The situation is more complicated for anisotropic nanoparticles such as nanostars or nanorods, for which it seems that SERS from single nanoparticles, especially from those molecules adsorbed on specific nanoparticle locations, is comparable to that of aggregates [29]. Still, at least in the case of spherical nanoparticles, which are the most commonly used, aggregation is needed to get a significant SERS effect. Spontaneous aggregation can be induced upon addition of the analyte solution, for different reasons. The analyte itself might readily adsorb in large amounts onto the nanoparticle surface, causing a sudden decrease of surface charge leading to the destabilization of the colloid, since the electrostatic repulsion between different nanoparticles is not enough to keep them apart anymore.

In the case of citrate-reduced colloids, for instance, since citrate ions are already present on the nanoparticle surface, a necessary condition to observe a SERS signal from an analyte is that it must be able to displace the citrate from the surface by strongly adsorbing on the metal. As a consequence, a major limitation of citrate-reduced metal colloids as SERS substrates is that their use with analytes bearing a net negative charge is problematic because of the analyte−particle repulsion. The addition of positively charged polyelectrolytes (e.g., polyamines) to the system usually helps in mediating the interaction between those analytes and the negatively charged colloids, working as an “electrostatic glue” between the two [36,37].

Ionic species, if present in the analyte solution, can shield electrostatic interactions, including those causing the repulsion between the colloidal particles, eventually leading to aggregation. However, it might be that the analyte is too diluted, and that the ionic strength of the solution is too low to induce a spontaneous aggregation, in which case some electrolytes (e.g., salts, acids, bases) can be purposely added to the system to induce aggregation.

Because of the electrostatic nature of the stability of the citrate-reduced colloids, for instance, these can be easily aggregated to maximize the SERS effect by increasing the ionic strength upon the addition of salts or saline solutions.

There are some circumstances, however, in which aggregation is hindered (e.g., by the presence of species that sterically stabilize the colloid, such as thick polymer coatings or layers of proteins around the nanoparticles) [38,39]. In those cases, the need for aggregation limits the use of most common colloidal

substrates. Often, to overcome problems related to aggregation, colloidal substrates are preaggregated (by adding small quantities of an electrolyte) before the addition of the analyte solution. In that case, small nanoclusters, or even dimers or trimers of nanoparticles are formed, forming the plasmonic nanogaps before coming in contact with the analyte solution.

6.2.2 Bottom-up noncolloidal substrates

Metal nanoparticles obtained by various protocols can be then assembled onto solid substrates, to form nanostructured surfaces that can be used as SERS substrates. Solid substrates used can be "hard" and compact solids such as silicon, quartz, or glass, or "soft" or porous such as polymers or paper. Simple, readily available and inexpensive substrates as glass and paper are often used. In particular, paper-based substrates (Fig. 6.3) are getting increasing attention: they are flexible, inexpensive, porous and allow the integration of chromatographic or microfluidics approaches to preprocess the sample before SERS analysis [40–43]. The nanoparticles dispersed in the colloid can be assembled onto the solid substrates by different methods, such as jet printing, spraying, drop casting, or dipping, usually leaving nanoparticles to self-assemble in random aggregates once the liquid medium evaporates. Sometimes, the nanoparticles are created directly on a solid substrate ("in situ nanoparticle synthesis") [44]. In all these cases, nanoparticles usually form

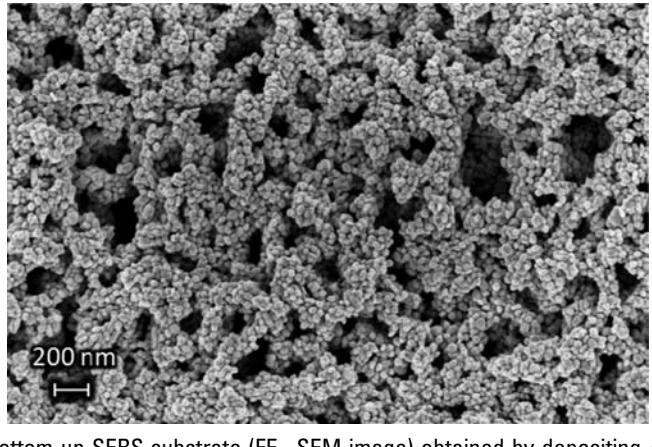

Figure 6.3 Example of bottom-up SERS substrate (FE–SEM image) obtained by depositing Au nanoparticles on a filter paper. *Source*: Reproduced with permission from Dalla Marta S, Novara C, Giorgis F, Bonifacio A, Sergo V. Optimization and characterization of paper-made surface enhanced raman scattering (SERS) substrates with Au and Ag NPs for quantitative analysis. Materials 2017;10:1365.

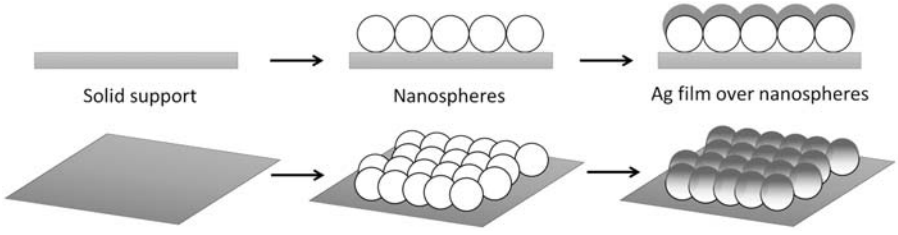

Figure 6.4 Schematic illustration of a process to create a SERS substrate (Ag—FON: film over nanospheres) with nanosphere lithography.

irregular, disordered nanostructures, and "hotspots" are irregularly distributed.

Another method that can be considered as "bottom-up" is nanosphere lithography [45]. In this method, polystyrene or SiO_2 nanospheres are deposited on a solid substrate (e.g., glass or silicon), forming ordered monolayers in which the spheres are regularly packed. Then, an Au or Ag layer is deposited on the top of these spheres (Fig. 6.4). The surface obtained is called Ag—FON (film over nanospheres), and its plasmonic properties make it an excellent SERS substrate. Alternatively, the spheres can be removed, leaving regularly spaced triangular metal nanoparticles where the interstitial spaces of the nanospheres layer were.

In general, the preparation of bottom-up noncolloidal substrates does not require special or particularly expensive instrumentation, while giving SERS substrates with good performance that can be used for in vitro diagnostic applications (see Section 6.3.3).

6.2.3 Top-down noncolloidal substrates

Top-down substrates can be approximately divided in two classes: substrates with ordered, regular surface structures and substrates with disordered, irregular, randomly arranged structures. Chemical etching of metal plates with strong acids is perhaps the simplest method to obtain roughened metal surfaces having irregular nanostructures. However, this method is not very reproducible and yields SERS substrates that are not very efficient. Another "chemical" method, which however requires the availability of an electrochemical setup, is the electrochemical roughening of metal surfaces with oxidation—reduction cycles [46]. This method leads to roughened metal surface with disordered nanostructures. Physical methods such as laser ablation can be also used to create SERS substrates with disordered features [47].

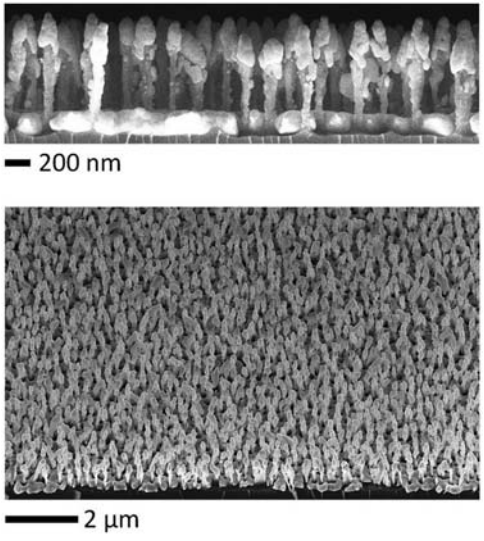

Figure 6.5 Scanning electron microscope images of a SERS substrate (i.e., Ag-coated Si nanopillars) prepared with a top-down approach. *Source*: Reproduced with permission from Schmidt MS, Hübner J, Boisen A. Large area fabrication of leaning silicon nanopillars for surface enhanced Raman spectroscopy. Adv Mater 2012;24:OP11–8.

Irregular surface nanostructures can also be created by physical or chemical etching methods on materials other than metal (e.g., silicon, Fig. 6.5), and then be successively coated with Ag or Au to obtain the plasmonic properties desired for SERS [48]. Ordered metal nanostructures, on the other hand, are usually obtained by using electron-beam lithography techniques [3].

6.2.4 Desirable characteristics

Besides an adequate EF, several other characteristics are important when considering the performance of SERS substrates. The most important characteristic for a SERS substrate is perhaps its reproducibility. In SERS, however, "reproducibility" is a complex, multifold concept, which is often misunderstood.

The reproducibility of a SERS substrate, for instance, might refer to the fact that spectra obtained on different substrates are qualitatively similar (i.e., they have bands at the same Raman shift and with the same "intensity pattern"). That is, their overall intensity can be different, but when normalized they are ideally identical. This kind of reproducibility can be easily accessed by collecting several spectral replicates (i.e., 5–10) of the same sample on different substrates and by evaluating the spectral variability (e.g., the standard deviation of the intensity of one or

more bands) after intensity normalization. As an option, this operation can also be done after the subtraction of a baseline. That is the minimum requirement for the reproducibility of a SERS substrate, allowing the development of methods for qualitative analysis or classification (e.g., identification or diagnosis). If an internal standard is used, this reproducibility will actually also allow the development of quantitative methods. This kind of reproducibility should not be taken for granted, since even small variations in the preparation protocol for the substrates, which might be due to different operators or to other experimental factors, can lead to small differences in the surface chemistry of the metal substrate, and thus to a slightly different analyte−metal interaction that will affect the SERS spectra.

An entirely different matter is the reproducibility of the overall intensity of the SERS spectra obtained from different substrates (i.e., the fact that two spectra can be overlaid even without normalization). This kind of reproducibility can be assessed by collecting several replicates of the same sample of different substrates and by calculating the intensity variability (e.g., the intensity standard deviation) of one or more bands, without performing intensity normalization. Also, in this case, this procedure can be also done after the subtraction of a baseline from all spectra, to compensate for differences in the background. The reproducibility in terms of absolute intensity is much more difficult to achieve, and it is very important when using the substrates for quantitative analysis.

Moreover, for noncolloidal substrates it is important to distinguish between intra- and intersubstrate reproducibility. For these substrates, spectra can in fact be collected from different areas or spots of the substrate, so that differences can be observed in spectra collected from different spots. The heterogeneity of a SERS substrate, both in terms of qualitative and quantitative response, can be assessed by collecting several replicates from different spots, or mapping an area of the substrates, and by calculating the spectral variability.

There is no general or standard procedure to assess the reproducibility of SERS substrates, but in any case all these aspects, and in particular, in the case of noncolloidal substrates, both the intra- and intersubstrate reproducibility should be considered.

Often, SERS substrates have adsorbates on their surface prior to the analyte addition. For colloidal substrates, these are the species conferring the colloidal stability (e.g., citrate), whereas for noncolloidal substrates some adsorbates can be present as a consequence of the preparation protocol, or as impurities. Thus

it is not uncommon to have substrates displaying a background signal that might interfere with the bands due to the analyte. For instance, all metal colloids prepared by citrate reduction have citrate ions ad adsorbates, yielding a characteristic SERS spectrum that might interfere with that of the analyte. A "flat" or low background, however, is certainly a desirable characteristic for SERS substrate, as it makes analyte detection easier.

A long shelf-life (i.e., the capability of retaining characteristics overtime) is also a distinct advantage for SERS substrate. Another desirable characteristic for SERS substrate, which will also impact on its shelf-life, is the stability toward a wide range of physical (e.g., temperature or light) or chemical (e.g., pH or ionic strength) conditions. Often, measurements must be performed in relatively harsh physical or chemical conditions, such as extremely acidic or basic pH. Citrate-reduced colloidal substrates, for instance, have a long shelf-life (months), but conditions as pH and ionic strength heavily impact on their stability, sometimes compromising the detection of a SERS spectrum.

Economic aspects such as substrate costs and their reusability are also important. In the development of many biomedical and diagnostic applications, a considerable number of independent spectra must be collected in order to build statistically significant predictive models. Thus the costs related to a single measurement cannot be too high, so that producing or buying the necessary substrates is feasible with a reasonable budget. The low costs are one of the reasons why paper-based substrates are gaining popularity, offering a reasonable trade-off between efficiency and cost.

While colloidal substrates can be used just once, noncolloidal substrates, at least in principle, could be "regenerated" to be reused more than once. Both physical (e.g., plasma cleaning or UV light) or chemical (e.g., exposure to strongly oxidizing or reducing agents) methods could be used to get rid of the organic matter present on the metal surface, without compromising the nanostructure itself [49–51].

6.2.5 Characterization techniques

6.2.5.1 Characterization of colloidal substrates

The simplest tool to characterize colloidal substrates is UV–visible absorption spectroscopy, yielding so-called "extinction spectra," which are the resultant of both scattering and absorption phenomena [52]. Extinction spectra give an indication of which are the plasmonic frequencies of the nanoparticles, but one has to remember that aggregated nanoparticles will behave

differently from individual ones, so that the extinction spectra will depend on the aggregation state, with aggregated nanoparticles showing a red-shifted maximum and a much broader band. Thus UV−vis spectroscopy is also useful to determine the aggregation state of your system. Moreover, so called “darkmodes” will be not visible from a far-field approach such as a UV−vis absorption experiment [53,54], but can play a significant role on the SERS performance of the colloids. For instance, spheroidal Ag nanoparticles display an extinction maximum between 390 and 420 nm, and, if aggregated the extinction maximum, will shift to the green or even to the yellow−orange part of the spectrum. However, such particles will display intense SERS spectra even when excited with a near-IR laser (e.g., at 785 nm), because of the occurrence of dark plasmonic modes around that wavelength [17].

Extinction spectra can also be used to check the shape of the nanoparticles: depending on the shape, nanoparticles can support more than one plasmonic frequency. For instance, nanorods show two extinction maxima, corresponding to two plasmonic frequencies: one frequency for each axis of the nanoparticles (i.e., short one and long one) [28]. Also nanostars show two extinction maxima, one for the “core” and one for the “spikes” of the particle [29].

Since extinction spectra depend on the shape and size of the nanoparticles, the width of the extinction band will give a gross indication of the size distribution of the particles. If nanoparticles have only one definite shape (e.g., spherical), then the narrower the width of the extinction band, the more monodisperse the colloid. This is not useful for precise and absolute analysis about size distribution, but rather to qualitatively compare the size distribution different colloid batches.

For spherical Au nanoparticles, thanks to some detailed studies [26], it is possible to use UV−visible extinction spectra to get a rather precise estimation of their size and concentration. However, for other shapes or metals, it is still necessary to use other characterization techniques to get a more precise estimation of nanoparticle size and concentration.

Although transmission electron microscopy is the safest and more accurate method to get information about the size and shape distribution of metal nanoparticles, less expensive and nondestructive methods based on light-particle interaction and Brownian motion analysis such as dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA) [55] can be also used. Moreover, zeta potential measurements, often combined with DLS and NTA are extremely useful. The zeta potential is

actually the potential difference, measured in V or mV, between the static layer of fluid around the nanoparticle and the bulk medium in which the nanoparticles are dispersed, but it can indirectly give an indication about the surface charge of the nanoparticles [56]. Usually, for electrostatically stabilized colloids (as most of SERS colloidal substrates), absolute values higher than 30 mV are indicative of a stable dispersion. Zeta potential measurements are also particularly useful for the qualitative determination of the surface charge, especially in those cases in which one aims at reversing this charge (e.g., from negative to positive) by substituting the adsorbed species forming the so-called "capping layer."

6.2.5.2 Characterization of noncolloidal substrates

In principle, noncolloidal substrates can be characterized with all the methods available in the field of surface science [57]. However, the most commonly used methods include scanning electron microscopy (SEM), often coupled with energy-dispersive X-ray spectroscopy (EDS), scanning tunneling microscopy, and atomic force microscopy. All of these can be used to get information about the substrate topology on the nanoscale, with SEM giving better results when investigating highly irregular surfaces such as those formed by random deposition of nanoparticle aggregates. EDS is also yielding information about elemental composition of the surface, including information about elements constituting the adsorbates. In EDS spectra, however, all molecular information about the adsorbed species is lost. Secondary ion mass spectrometry can give more information about adsorbed molecules, since charged molecular fragments are detected.

Plasmonic frequencies arising from opaque, optically dense noncolloidal substrates can be investigated with UV–vis–NIR diffuse reflectance spectroscopy. This technique works particularly well with highly irregular and porous surfaces, such as those of nanoparticle-on-paper substrates [58].

6.3 Surface-enhanced Raman spectroscopy substrates for bioanalysis, diagnostics, and theranostics

There is no such thing as a "general" SERS substrate that can be used with any analyte. Since the SERS response is the result of a complex interplay between the analyte, the matrix, and the

metal substrate, each analytical problem requires a SERS substrate with its own proper characteristics. It is highly advisable to choose the metal nanostructure in function of the analytical problem, and of the overall strategy chosen to tackle it. Biological samples such as biofluids (e.g., plasma, serum, urine, and saliva) are chemically complex mixtures, often containing several thousands of chemical species [59,60]. Sometimes the goal is to obtain a biochemical fingerprint of a biological sample, without specifically targeting one analyte, but aiming to get as much information as possible from SERS spectra, thus hoping to detect as many biomolecules as possible. This is called an "untargeted" approach. In other cases, one is interested in a specific analyte. Detecting or quantifying a specific analyte amidst all the biochemical species constituting the biological matrix, without a separation step involving a chromatographic approach, is a formidable task, requiring a definite strategy and, accordingly, a substrate with suitable properties. The SERS substrate must meet at least two requirements: it should have a plasmonic response at the wavelength selected for excitation, and it should be able to capture or bind the analyte of interest.

6.3.1 Indirect versus direct surface-enhanced Raman spectroscopy detection

The first and most important aspect to define in order to design or select a suitable substrate is the choice between a direct detection and an indirect detection strategy (Fig. 6.6). A direct detection of the analyte involves the direct sensing of the vibrational bands due to the analyte or analytes of interest, whereas in the indirect detection, the presence or quantity of the analyte or analytes is inferred from the variation in intensity or Raman shifts of bands due to vibrations of other molecules (probes). The main challenge in the direct detection strategy, especially when the matrix in which the specific analyte of interest is found is chemically complex (e.g., a biofluid), is to limit the interference from all the other chemical species, which will compete with the analyte for the adsorption onto the metal surface (see Section 6.3.2). Usually, unless the analyte itself has a very good affinity for the metal surface, the direct detection of a specific analyte in a complex matrix is very challenging. Lowering the complexity of the matrix (e.g., by introducing some preprocessing steps such as analyte extraction) or modifying the surface to make it more attractive for the analyte are two possible options. Strategies for surface functionalization

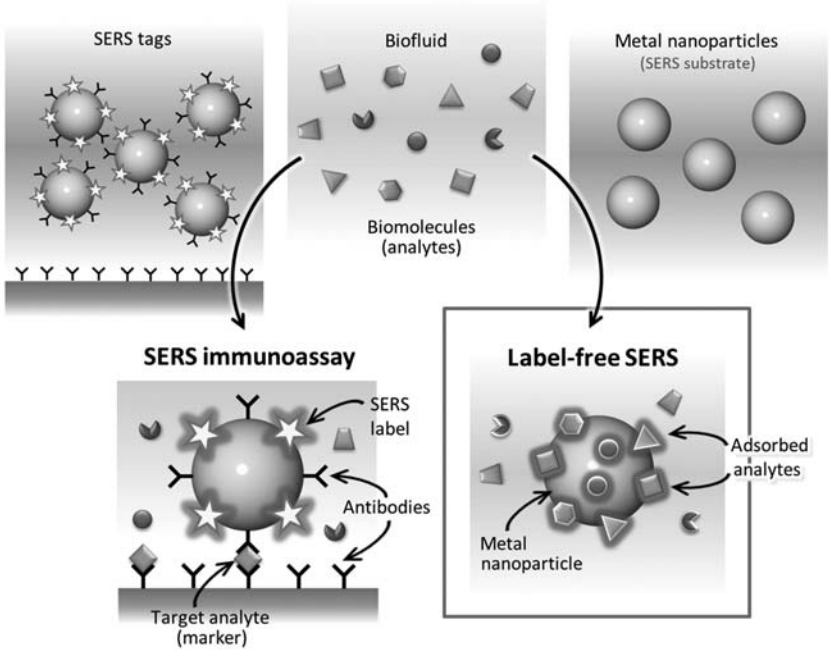

Figure 6.6 An example of indirect (on the left) and direct (on the right) detection strategies for SERS of biofluids using colloidal substrates. *Source*: Reproduced with permission from Bonifacio A, Cervo S, Sergo V. Label-free surface-enhanced Raman spectroscopy of biofluids: fundamental aspects and diagnostic applications. Anal Bioanal Chem 2015;407:8265–77.

include the modification of the surface charge or hydrophobicity, such as using self-assembled monolayers. Alternatively, the analyte can be forced to bind close to the surface by a chemical reaction causing the formation of a bond between the analyte and a small molecule immobilized on the metal surface. In any case, the surface functionalization should not increase too much the distance between the analyte and the surface; otherwise, the SERS effect, which rapidly decreases with the distance from the metal, will be negligible. If the surface has physicochemical characteristics (e.g., surface charge) that are compatible with the analyte of interest, the direct detection usually does not require further substrate functionalization. The direct detection strategy, since it is carried out without the use of Raman reporters or labels, is usually referred to as "label-free." An "untargeted," label-free approach is also possible, to retain as much as possible the biochemical complexity of the sample. In this approach, one does not look for a specific analyte but for as many biomolecules (e.g., metabolites) as possible, so that the interference from the matrix becomes less of a problem.

On the other hand, a “indirect detection” approach usually requires substrate functionalization. Raman reporters or labels are used in order to reveal the presence of the analyte, and they can be used according to different strategies. A common strategy is to bind both Raman reporters and recognition elements (e.g., antibodies) to nanoparticles, obtaining objects that are often called “SERS nanotags” [61]. These SERS nanotags can be used in tests in which the target analyte is first captured by recognition elements on a different substrate, and then the nanotags, upon binding to the target, are used to reveal if the analyte is present. Another strategy is to use SERS beacons (i.e., molecular systems that can vary the distance between a Raman reporter and the metal surface in presence of the analyte) [62]. A different approach consists of using chemical reactions between a Raman reporter bound on the surface and the analyte, while looking for changes in the signal of the reporter [63,64]. Often, Raman reporters are dyes in resonance with the exciting laser, so that the further enhancement given by SERRS can be exploited [65,66].

Glucose is an example where both direct and indirect approaches have been tried. The direct approach has been challenging, since glucose has little affinity for gold or silver surfaces, so that metal surfaces must be functionalized with self-assembled monolayers capable to bind the sugar [67,68]. In the indirect approach, glucose was captured by organoborates via a chemical reaction, causing changes in the spectrum of these molecules that were clearly detectable, allowing the indirect sensing of glucose even in biological media [63,64]. These results were obtained using noncolloidal substrates, which allowed better surface functionalization and avoided problems related to interference from other molecules present in the biological media.

6.3.2 The role of the nanobio interface

When considering bioanalytical SERS applications using biological samples, one has to carefully consider the biochemical complexity of the biological matrix. When a nanostructured metal substrate is put in contact with a biological sample such as a biofluid, many biomolecules will spontaneously adsorb on the metal surface, creating a complex system called the nanobio interface [69]. The nanobio interface has been well characterized in the case of gold nanoparticles and blood or cells, especially as far as proteins are concerned [70,71], but information about this interface in the case of other nanostructured gold

and silver surfaces or other biological samples is rather limited. For Au nanoparticles, as soon as these nanostructures enter in contact with a protein-rich biological environment, such as blood or cytoplasm, a layer of adsorbed proteins called “protein corona” rapidly forms [70]. A similar layer also forms on non-colloidal metal surfaces, which, depending on the specific application, may impair their function as SERS substrates (“protein fouling”) [72]. Besides proteins, a plethora of low-molecular-weight biomolecules can strongly adsorb on the metal surface, possibly interfering with the SERS detection of an analyte. In general, when looking for a specific analyte with SERS, the formation of a nanobio interface can cause two types of problems. First, it can lead to saturation of the substrate surface by other biological molecules, impeding the adsorption of the analyte on the metal surface. Second, even if the analyte has an affinity and concentration allowing it to coadsorb together with the matrix biomolecules, the signal of the latter may strongly interfere with the bands due to the analyte, de facto hindering its detection, especially in the case of a direct detection strategy. This is true for all kinds of SERS substrates, but for colloidal substrates there is another major problem with the formation of a protein corona: the hindering of colloidal aggregation by steric stabilization of the nanoparticles. Since colloidal aggregation is functional for the formation of SERS active sites, biological samples with a high protein concentration (e.g., blood serum or plasma), by promptly forming a protein corona around nanoparticles, may yield weak SERS spectra, or no SERS spectra at all. Thus for protein-rich samples a deproteinization step (e.g., by filtration) is often required to obtain intense SERS spectra from colloidal substrates. A preaggregation step, e.g., the addition of an aggregating agent or by increasing nanoparticle concentration by centrifugation, is also an option to overcome the problems caused by the protein corona [73].

Protein corona and protein fouling, however, are only part of the problem, and they can solved by methods such as deproteinization or, in the case of noncolloidal substrates, by functionalization of the surface with an antifouling coating. From SERS data, we know that in many cases (e.g., plasma, serum and cytosol) low-molecular-weight molecules are strongly adsorbing on the metal surface, forming a “small-molecules corona” [73−76]. These molecules, mostly purines and −SH containing molecules (e.g., glutathione), can saturate the available sites on the metal surface, or can yield such strong SERS signal to obscure the signal due to the analyte, especially when a direct

detection strategy is employed. Moreover, the variability of the biological matrix signal (e.g., the interindividual variability in the case of blood or urine samples) often makes a univariate data analysis, where the intensity or area of a single band is considered, unfeasible, in favor of a multivariate approach. A possible solution to this problem is to functionalize the metal surfaces with a layer having the two-fold function of protecting the surface against the unwanted adsorption of small molecules of the matrix and of promoting the adsorption of the analyte [77]. Such a functionalization is nontrivial: among others, the use of molecularly imprinted polymers was suggested [78,79] as a possible strategy.

6.3.3 Surface-enhanced Raman spectroscopy substrates for in vitro diagnostics

Both colloidal and noncolloidal SERS substrates can be used for in vitro diagnostics, with both direct and indirect detection strategies. Samples such as biofluids, especially serum or plasma, which are rich in proteins, might constitute a problem for analytical strategies using colloidal substrates and requiring aggregation (see Section 6.3.4), while noncolloidal substrates might incur in the problem of protein fouling. Biofluids can be directly deposited on noncolloidal substrates, but then they must be left to incubate for some time and be washed away, or let dry. In the latter case, depending on the drying conditions, the sampling area can become extremely heterogeneous, with different parts of the substrate yielding different spectra, to the detriment of experiment repeatability. Moreover, depending on the volume of biofluid, the drying process can take some time, from 15 to 30 minutes (for few microliters) to more than 1 hour. On the other hand, colloidal substrates require a "mixing" step with the biofluid sample, but then the resulting mixture can be immediately measured without delays. SERS substrates, from a point-of-care (POC) perspective, can also be incorporated into lateral flow assays devices [80–82], so that sample preprocessing or separation steps can be performed on the sample before it reaches the substrate (Fig. 6.7). Colloidal substrates deposited or ink-jet printed on paper can also be part of a so-called "paper analytical device" [83]. These devices are single-use analytical platforms on small pieces of paper, onto which polymers or waxes are printed to design microfluidic channels for separation, mixing, or other preprocessing steps for the sample before SERS detection. These paper-based SERS devices are

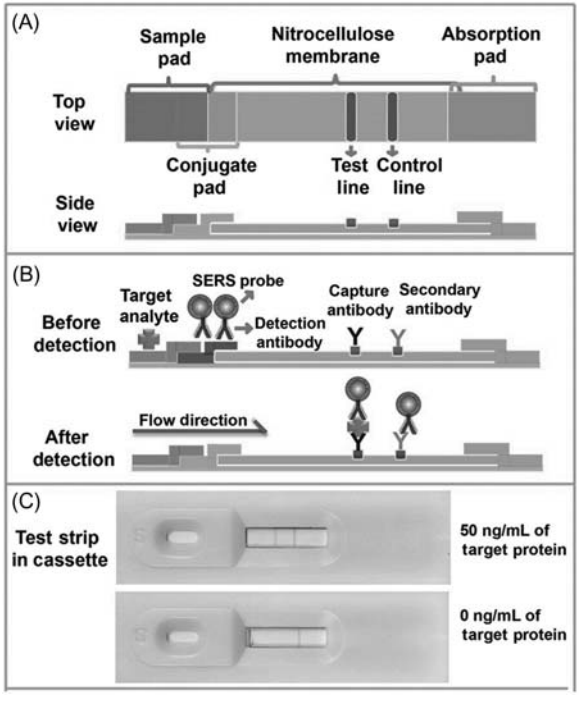

Figure 6.7 Schematic illustration of the operation principle of SERS paper-based lateral flow strip (PLFS). (A) Top and side views; (B) side view before and after biomarker detection; (C) optical photos of PLFS assembled in cassettes in the presence (upper) and absence (bottom) of the target. *Source*: Reproduced with permission from Gao X, Zheng P, Kasani S, Wu S, Yang F, Lewis S, et al. Paper-based surface-enhanced Raman scattering lateral flow strip for detection of neuron-specific enolase in blood plasma. Anal Chem 2017;89:10104−10.

particularly attractive for diagnostic applications in a clinical setting or even from a POC perspective, since they are affordable, robust, and easy to manage and use.

If the in vitro diagnostic test has to be performed on cells, the type of substrate to be used depends on the analytical strategy used. Usually, SERS nanotags labeled with a Raman reporter are used to detect specific cells with an indirect detection strategy, as in the case of circulating tumor cells [84,85]. These tags are meant to bind the external cell membrane of specific cells, revealing their presence in the sample. More recently, other approaches to characterize cells were proposed, such as the analysis of cell lysates [75,76] using colloidal substrates, or the analysis of the cell secretions (also called SERS optophysiology) using noncolloidal substrates [86]. These two approaches, however, have been proposed as methods to characterize cells and still need to be tested as diagnostic methods.

The use of metal nanoparticles as label-free sensors inside intact cells (i.e., after an active or passive uptake) is also possible [87–90], but results reported by different studies are rather heterogeneous and no diagnostic applications have been reported yet.

6.3.4 Surface-enhanced Raman spectroscopy substrates for in vivo diagnostics and theranostics

The type and characteristics of a SERS substrate to be used in vivo must be selected according to its purpose: SERS can be used for the direct sensing of a specific analyte (e.g., glucose levels in the blood) or for the disease detection in terms of spatial localization. Usually, the latter is achieved by using SERS nanotags to define where the diseased tissue is spatially located. In this sense, the intense signal due to SERS nanotags is used as a contrast agent for imaging. This approach can be used in diagnostics, to detect and locate the diseased tissue in the body, using a spatially offset approach [91] for regions relatively close to the body surface or coupled to endoscopy [92] to reach inner tissues. The same approach can even be used intraoperatively to guide the surgeon in defining the margins on the diseased tissue to remove [93]. In all these cases, the design of the SERS nanotag is guided by the same principles (Fig. 6.8), and the choices to be made strictly depend on the final application.

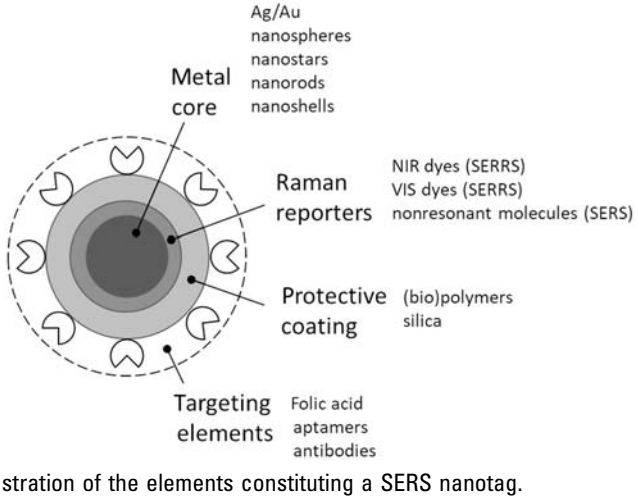

Figure 6.8 Schematic illustration of the elements constituting a SERS nanotag.

Au is mostly used as metal for SERS nanotags to be used in vivo, because of its lower chemical toxicity with respect to other SERS metals (e.g., Ag or Cu). Bulk Au is chemically inert and a-toxic. Still, nanomaterials such as nanoparticles can display a toxicity related to the size and shape of the material rather than on its chemical composition. This aspect is still being investigated for Au nanoparticles, so that toxicity still remains a concern [61]. For this reasons, diagnostic approaches based on SERS using topically applied nanoparticles (e.g., in combination with endoscopy [94,95]) are considered safer with respect to those requiring intravenous administration of nanoparticles.

The morphology of the nanoparticles is important in defining its plasmonic properties, and thus which laser can be used to get a SERS effect. In general, biological tissues are more "transparent" in a spectral region going from the red to the near-IR [96], so Au nanoparticles with morphologies such as nanostarts, nanorods or hollow nanospheres, having extinction maxima in those regions, are preferred. Moreover, these nanoparticles can efficiently convert the absorbed light into heat, making them ideal candidates for photothermal therapy applications combined with diagnosis (i.e., theranostics) [97−101].

Raman reporters to be embedded in SERS nanotags for in vivo applications must give a signal as strong as possible: ideally, dyes absorbing in the NIR should be used [96], to exploit a SERRS effect (see Section 6.1.4), maximizing the signal intensity. To prevent the release of these potentially toxic Raman reporters into the organism, as well as to protect them from unwanted accidental desorption due to potentially aggressive biological environments, a protective coating layer, made of polymers, proteins, or silica, is used [61].

The SERS nanotags must reach and accumulate in the diseased tissue via passive or active mechanisms. Nanoparticles can passively accumulate in the diseased tissues, but most often active targeting strategy is to be preferred, by functionalizing the SERS nanotag surface with specific targeting elements such as antibodies, folic acid or aptamers [61].

When the purpose of in vivo SERS sensing is the detection of a specific analyte (e.g., glucose), different strategies are employed, involving the use of noncolloidal substrates. Implanted solid SERS substrates [102,103], patches with intradermal microneedles [104,105], or macroscopic needles with a nanostructured tip or surface [1,106,107] have been used for this purpose. In these cases, toxicity is no longer a major concern, whereas the challenge is to keep the substrate "active" for a longer time,

preventing its degradation due to the interaction with the biological environment and/or the irreversible saturation of its sensing surface with the analyte. A proper surface functionalization, by protecting the metal surface while ensuring a reversible analyte trapping, can play a crucial role in solving these problems [61], but, as in other SERS applications, there is still no general solution, and each analytical problem must be specifically addressed.

6.4 Concluding remarks and perspectives

SERS substrates are complex objects addressing a complex function, and their design necessarily requires interdisciplinary expertise. Plasmonic aspects have to be considered according to specific physical models; surface functionalization requires a careful chemistry, and the coating with targeting molecules or recognizing elements involves a biological knowledge of the disease involved. In this sense, designing a SERS substrate for bioanalysis perfectly embodies the intrinsic multidisciplinary nature of nanotechnology.

Because of their complexity, SERS substrates must be tailored to the specific bioanalytical problem: experimental details such as the wavelength of the laser to be used, apparently less relevant, are extremely important in defining many aspect of the substrate, so that nothing is left to chance.

Perhaps the most important decision to be made when planning the development of a SERS substrate for bioanalytical purposes is its final use: will it be used in vivo or in vitro? For in vivo substrates, the options are limited, whereas in vitro detection allows for a broader variety of choices. Then, another crucial decision is the strategy to be adopted: direct versus indirect detection. This decision will have consequences over all the other aspects, from the nature of the metal to be used (and then, as a consequence, the type of laser to be used) down to the complexity of the surface functionalization. In all cases, the interplay between the nanostructured metal surface and the incredibly complex and rich biological environments, be it that of biofluids, of tissues, or of cells, must be reckoned with. To summarize: selecting or designing a SERS substrate for diagnostic or theranostic purposes is far from trivial, and it is a task requiring a considerable amount of effort, including careful planning of the strategy to be used.

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