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Identification of organic dyes by surface-enhanced Raman scattering in nano-composite agar-gel matrices: evaluation of the enhancement factor

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Abstract The aim of this work is to improve our understanding of the new possibilities offered by a micro-extraction/surface-enhanced Raman scattering (SERS) identification method for organic dyes used in artworks. Recently, we have shown how it is possible to use a tailor-made extraction process of the dyes present on different kind of substrates by using a nanocomposite hydrogel matrix containing silver nanoparticles, without detriment for the original samples. After minor processing, we identified the extracted dyes thanks to the clear bands in their SERS spectrum, and the comparison with data on reference materials. A usual drawback in SERS/Raman experiments is the lack of information on the sensitivity of the method. Sometimes, quantitative information in SERS is obtained, mostly while working in solution, either with samples of known concentration of the analytes or in presence of an internal standard. Here, in order to obtain similar information on our samples, some specific methods are applied to gain information on the SERS enhancement factor for a reference dye molecule. We report new data on the efficiency of the SERS

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process for the identification of organic dyes extracted by an agar hydrogel matrix containing silver nanoparticles.

Keywords Raman · SERS · Enhancement factor · Dyes · Textiles

1 Introduction

Color has always been the most important feature in man-made materials. Therefore, the identification of the different dyes and pigments used in the preparation of any artistic object is mandatory in order to fulfill a number of different tasks when studying the object itself or its relation with the human society that created or used it. Scientific approach to conservation requires the knowledge of the material in order to plan and execute any conservative treatment on the samples. At the same time, the art historians are well interested in the identification of the specific materials used by the different artists. Given the relevance of the artworks in the building of the specific identity of any human society, the possibility to follow the diffusion of dyes and pigments along the different territories and ages is a very good way to trace the development of trades, technical, and cultural exchanges between different populations and lands.

In the last years, a lot of activity has been devoted to the specific and sensitive identification of dyes and pigments. Perhaps, the most challenging one has been the development of methods for identification of organics dyes, due to their susceptibility to the chemical and physical environment and to the presence of different, but chemically quite similar, relevant molecules in the natural dyes extracted from plants or animals. Ideally, the identification method of choice to study precious artworks (or historical samples) should provide highly specific information, with high sensitivity, without sample detriment. At present, the most accurate information is obtained by the extraction of dyes with suitable solvents and the analysis of extracts with high performance liquid chromatography (HPLC) or, even better, HPLC coupled to determination of the different components in the extracts by mass spectrometry (HPLC–MS) (Degano et al. 2009; van Bommel et al. 2007). However, this approach has a couple of major drawbacks. The first is the use of “hard” methods for the extraction of the dyes that destroys a (small) portion of the artwork. The second is the risk, in the long and—sometimes—involved extraction procedure, to chemically transform or lose part of the extracted dyes. In the last years, it has been shown that optical spectroscopy methods can be fruitfully applied for the identification of coloring materials (Burgio and Clark 2001; Casadio et al. 2010). Color and electronic absorption/reflectance spectra can be easily determined with very good sensitivity and without the need of any sample treatment. However, the information is not accurate with respect to the chemical composition of the sample (Berrie 2012). Much better specificity is possible when vibrational spectroscopy methods are applied (Burgio and Clark 2001). Among the different vibrational spectroscopy methods, Raman spectroscopy has proven to be the best performing one for a number of reasons, including the possibility to operate in a non-destructive way, without sample treatment and with high spatial selectivity on inhomogeneous materials. Raman spectroscopy suffers for two major constrains that are its limited sensitivity and the possible interference with fluorescence emission. These limitations are quite relevant when studying organic dyes, as those used in a number of different applications, from the dyeing of textiles already in the very early days of human history with natural organic molecules, to the contemporary and widespread use of modern synthetic dyes. Actually, these organic molecules are applied in very scanty amounts, given their very high tinting power. In addition, they exhibit a strong fluorescence emission upon excitation in the visible part of the optical spectrum (the usual experimental condition for Raman spectroscopy measurements). Quite recently, different groups have devised various strategies to overcome these problems for the identification of dyes in artworks (Leona 2009; Platania et al. 2014; Idone et al. 2014; Doherty et al. 2011). The general approach was to use the interaction of the dye molecules with a nanostructured metal surface, typically silver nanoparticles (AgNPs), in order to get a strong enhancement of the Raman scattering process (SERS) and, at the same time, to quench the fluorescence emission efficiency. The NPs cannot be sprayed (or transferred by other simple methods) on the historic manufacts, as their contamination must be avoided. Thus, methods have been developed aiming either to do some limited sampling of the dye and then perform SERS experiments (Leona et al. 2006; Platania et al. 2014) or to apply reversibly the NPs to the sample, e.g. by applying a suitable peelable coating to the sample during the experiments (Doherty et al. 2011). The subject was reviewed recently (Casadio et al. 2010; Muehlethaler et al. 2015; Ricci et al. 2016a).

SERS enhancement is dominated by the amplification of light by the excitation of localized surface plasmon resonances occurring on nanometric singularities on the metal surface (like sharp features or gaps), which is a physical process (Mock et al. 2002; Stiles et al. 2008). The corresponding signal enhancement, in most case, scales with the fourth power of the field enhancement (Le Ru and Etchegoin 2006). Also, chemical processes can be involved in SERS, such as resonant excitation of cluster↔molecule charge-transfer excitation or intramolecular electronic excitation. The overall SERS enhancement is the product of the different enhancement factors. Recently the calculation of this enhancement factor was performed, using different approaches (Zhao et al. 2006; Yurkin et al. 2007; Lofrumento et al. 2016b). In principle, this enhancement can exceed a 10^{11} factor and can lead to single molecule detection (Kneipp et al. 1997; Le Ru et al. 2006; Dieringer et al. 2007). The subject has been reviewed by different authors (Kneipp et al. 2006; Aroca 2007; Le Ru and Etchegoin 2009; Schlücker 2011).

We have developed a method for "soft" extraction of dyes from textile fibers or other supports and we have shown its possible application for their identification by SERS spectroscopic methods (Lofrumento et al. 2013; Platania et al. 2014, 2015). However, this approach is completely general and it has been recently applied in different fields of research, like in food analysis for the detection of pollutants or dyes on the surface of fruits (Gong et al. 2014, 2016; Lin et al. 2014).

In our work on dyes present in ancient textiles, we use a small agar–agar hydrogel cube (typically 5 mm side), loaded by a suitable chemical reagent to facilitate extraction of different types of dyes, like mordant and vat dyes from the samples (Lofrumento et al. 2013; Platania et al. 2014, 2015). By testing the process both on mock-up and on real samples, with a careful selection of chemical agents, we have shown that we can extract by the gel a small amount of dye, which is large enough for dyes' identification by SERS measurements, but it is small enough that no detectable change in color occurs. The nature of the extracting chemicals is tuned both to the specific properties of the dyes and to the procedures used for the tinting of the fabric. AgNPs were added to the hydrogel either during its preparation (by using directly the colloidal dispersion of AgNPs to dissolve the agar–agar) or after the extraction process, by putting a few drops of AgNPs colloidal dispersion in top of the gel cube, on the site that was in contact to the sample. Once we remove the hydrogel cube from the contact with the fibers, we let it to dry in air at room temperature; it loses 90–93 % in weight and collapses leading to an effective concentration of the analytes. Then we characterize the extracted molecules present in the dried sample by SERS spectroscopy. From the experimental point of view, it is quite easy to observe a strong increase in the Raman signal from these samples but even simple quantitative evaluations are rather difficult given the nature of the enhancement process and the sample preparation process.

In order to have some insight on the sensitivity of the method, we propose to perform a systematic study on the changes of the Raman signal of a reference dye by looking at different samples (solutions, powders and dried gels) containing the dye alone or in presence of AgNPs.

The characterization of systems in solution allows for a clear experimental determination of the SERS enhancement efficiency, at molecular level, under some assumptions on the description of the system. The presence of an internal standard, or the comparison of signal intensities measured on concentrated solution (Raman) and for dilute solution (SERS), is used for this purpose (Le Ru et al. 2007). More complex is the problem when dealing with ill-defined systems, like powders (obtained from evaporation of the reference solutions) or dried gels.

In this work we shall present results on the SERS enhancement factors in solution and in these condensed phases, for an organic dye, *o*-Safranin (3,7-diamino-2,8-dimethyl-5 phenylphenazonium chloride, abbreviated as SO). SO is a cationic azinic dye that strongly absorbs blue-green visible light (peak absorption at 515 nm). At present, it is used in biology for cell staining, in dye-based solar cells and as a photosensitizer in electron/energy transfer reactions. In earlier times, it was used for the production of cinematographic films as a sensitizer or as a toning color Lofrumento et al. 2016a. It was chosen as its spectroscopic properties (UV–Vis electronic absorption, Raman and SERS) are well known and it has a reasonably good water solubility (Lofrumento et al. 2015; Ricci et al. 2016b).

2 Experimental

Raman spectra were obtained by using an assembled micro-Raman spectrometer equipped with a He–Ne laser operating at 632.8 nm, a Jobin–Yvon HR460 spectrometer equipped with 600 lines/mm grating and a Spectrum One liquid nitrogen cooled CCD camera (Ricci et al. 2016b). An optical Notch filter (SuperNotch, Kaiser Optics) filtered the scattered radiation before entering the spectrometer to suppress effectively the elastically scattered excitation radiation. We performed the observation of the samples and the scattering experiments through a modified Olympus BH2 microscope (with 50× and 10× objectives). Raman and SERS scattering experiments were carried out in backscattering geometry. Working on solids, we used a 50× objective that focuses the laser radiation down to a 2 μm diameter spot while experiments on solutions were performed with a 10× objective. We measured the laser power at the sample with a calibrated silicon diode detector (FieldMaster, Coherent). The Raman experiment was carried out in preresonance conditions (electronic absorption around 515 nm, Raman excitation at 632.8 nm—see Fig. 1). This allows for a rather good sensitivity in the Raman spectrum while fluorescence is not exceedingly strong with respect to the Raman signal. Another experiment carried out in full resonance condition (excitation with the 514.5 nm line of an argon ion laser) has

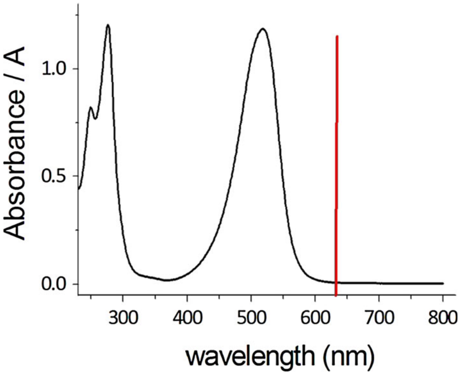

Fig. 1 The electronic absorption spectrum of a 10^{-4} M SO solution in water (10 mm optical path). The 632 nm excitation wavelength used for the Raman scattering experiment is indicated by the *vertical red line*

shown that in solution the Raman spectrum is completely covered by the very strong fluorescence background.

Silver nanoparticles (AgNPs) were prepared in colloidal dispersion following the standard Lee–Meisel method (Lee and Meisel 1982). In particular, after a reaction time of 60 min for the chemical reduction of the Ag^+ ions, the flask containing the colloidal solution was placed in an ice bath to cool. This prompt cooling process of the colloidal dispersion has been adopted in order to obtain a narrower distribution of nanoparticle sizes having an average diameter of about 40 nm. Their physical properties were characterized by the measure of the extinction and/or absorption spectrum of the colloidal solution because the wavelength of the maximum extinction peak, its width and intensity refer to the dimension and concentration of the metal nanoparticles (Solomon et al. 2007). The relative values of the absorption and extinction coefficients refers to the nature of their interaction (absorption and scattering processes) with the electromagnetic waves (Feis et al. 2014). TEM microscopy was also used to verify the average diameter of the AgNPs.

The agar gel was prepared by using common grade agar–agar in flakes. The flakes were washed repeatedly with distilled water in order to remove any traces of chloride ions and then they were dried and ground. For the preparation of the agar gel, 2 g of agar–agar were mixed in a beaker with 100 mL of water. The sol–gel mixture was heated in a microwave oven at 300 W for a couple of seconds. Then, the viscous solution was poured into a Petri dish and left to cool down to room temperature. Finally, small cubes were cut with a calibrated tool.

Samples in solution were prepared by mixing in a glass vial appropriate amounts of SO dissolved in water and the colloidal dispersion containing the AgNPs. The solid samples were prepared by evaporation on a glass slide of either 8 µL of 10^{-4} M SO solution in water (Raman) or 8 µL of a mixture containing 4 µL of the AgNPs colloidal dispersion and 4 µL of 10^{-4} M SO solution in water (SERS). The gel samples were prepared by adding in top of the fully hydrated agar gel cubes (5 mm side) either 8 µL of 10^{-2} M SO solution in water (Raman) or 8 µL of a mixture containing 4 µL of the AgNPs colloidal dispersion and 4 µL of 10^{-4} M SO solution in water (SERS). Alternatively, the gel sample for SERS was prepared by using the AgNPs colloidal dispersion as the solvent in the hydrogel preparation, then, the cubes made out of this gel were added with 8 µL of a mixture containing 4 µL of the AgNPs colloidal dispersion and 4 µL of 10^{-4} M SO solution in water.

3 Results and discussion

The extinction spectrum of the colloidal dispersion containing our AgNPs exhibits a marked maximum at 415 nm that is associated with a size distribution of the particles centered at 40 nm diameter (Solomon et al. 2007). The analysis of TEM and Helium ion scattering images of different samples of these NPs supported this result (Platania et al. 2014, 2015). Commonly, these AgNPs find an application as efficient substrates for SERS experiments given the simple preparation methods and stability. It is therefore quite interesting to evaluate their effective efficiency in promoting the Raman scattering process. This efficiency is measured by the so-called (molecular) SERS enhancement factor EF^{SERS}, which is expressed as.

$$EF^{SERS} = \frac{I^{SERS}}{I^{RS}} \times \frac{N^{RS}}{N^{SERS}}$$

that is the ratio between the intensities I of the SERS and Raman signals weighted by the relative number N of molecules involved in the two processes (the label *RS* indicates the quantities relative to the Raman signal, *SERS* those related to the SERS signal) (Le Ru et al. 2007; Murcia-Mascarós et al. 2005). Therefore, we need to evaluate the number of molecules involved in both processes, together with that of the SERS and Raman signals, from each sample (liquid, solid, and gel phases).

We calculate the amount of SO available in solution just by taking into account the molar concentration of the water solutions we prepared. The number of AgNPs involved in the process is evaluated through a spectrophotometric measurement. A molar extinction coefficient of 8×10^9 M^{-1} cm^{-1} was reported for this kind of AgNPs colloidal dispersions (Evanoff and Chumanov 2004). Given the measured 1.5 value of extinction over a 2 mm optical path-length, an AgNPs concentration of 1.0×10^{-9} M is calculated.

We will continue our discussion under the hypothesis that the SO molecules, whenever possible, cover the AgNPs as a monolayer. Assuming, by simple molecular modeling, that each SO molecule covers an area of 7×10^{-1} nm^2, each AgNP of 40 nm diameter can interact with a maximum of *approx.* 7000 molecules. Therefore, our determination represents a lower limit for the SERS enhancement factor EF^{SERS}.

The SO water solution for Raman measurements was 10^{-2}M. The SO solution for SERS measurement was prepared by mixing equal amounts of an 1.0×10^{-4} M solution of SO in water and the 1.0×10^{-9}M colloidal dispersion of AgNPs. In this sample, given the ratio of the molar concentration of SO and AgNPs, the SERS signal intensity is limited by the number of AgNPs and the concentration of SO molecules contributing to SERS signal is only 3.5×10^{-6} M. We show in Fig. 2 the SERS and Raman spectra measured in solution. We measure in these spectra the intensities of the four major peaks (marked in the figures) and we introduce in the above expressions for the SERS enhancement factor EF^{SERS} the average value of their intensity ratios. A direct evaluation of these numbers leads to an EF^{SERS} in solution is as large as 220000 ± 50000. However, as the color of the 10^{-2} M SO solution is very dark, we must correct the raw Raman intensity data for the optical transmission of the solution at the different wavelengths. We measured the absorption spectrum of the 10^{-2} M SO water solution in a 2 mm thick optical cell. In the relevant spectral regions for the Raman spectrum we have shown, we obtain the following results: 0.65 A at 633 nm (excitation wavelength) and 0.2–0.08 A in the region between 655 and 710 nm (corresponding to a 500–1700 cm^{-1} Raman shift). The 10^{-4} M SO solution in water or in the colloidal dispersion with AgNPs have a negligible absorption in this

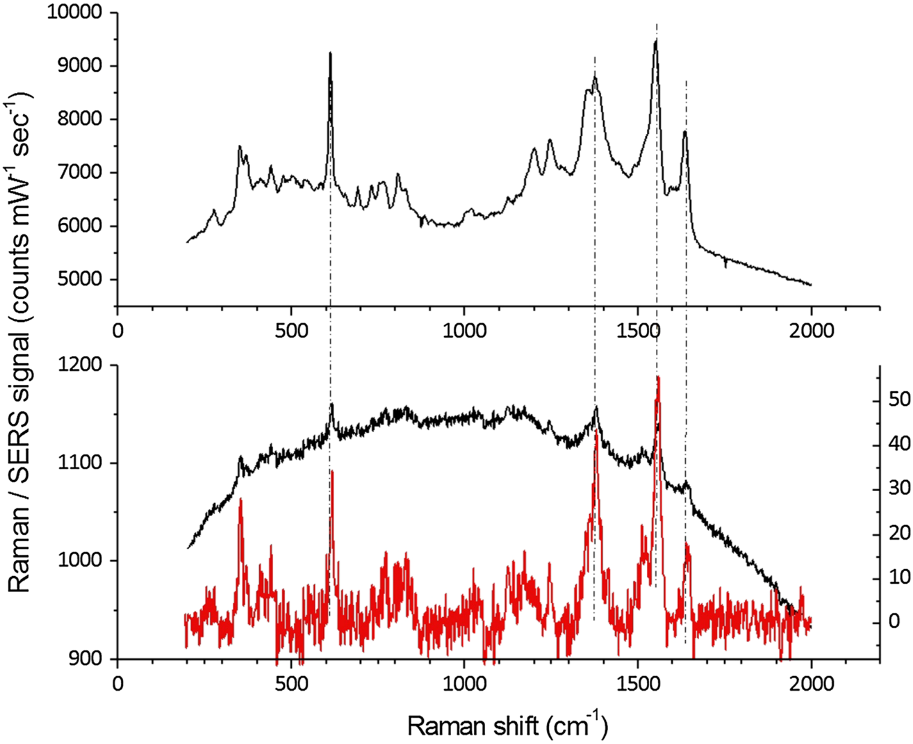

Fig. 2 The Raman spectrum of a 10^{-2} M solution of SO in water is shown on the *bottom panel*, both as raw data (*black trace*, left axis) and as background subtracted data (*red trace*, right axis—units: counts mW^{-1} s^{-1}). The SERS spectrum of 5 × 10^{-5} M SO in the colloidal dispersion containing AgNPs (see text for details) is shown in the upper panel. We used the four bands connected by vertical lines for the calculation of the SERS enhancement factors. Experimental conditions: excitation at 632.8 nm, laser power 1.0 mW at the sample, microscope objective ×10

spectral range. The laser spot on the small glass tube containing the solutions under the microscope was approximately 2 mm below the glass surface. Then, using the data reported above this correspond to an overall attenuation factor of 7–8 of the Raman signal obtained from the 10^{-2} M SO solution due to the absorption of both the excitation and emission radiation. Therefore, the corrected SERS enhancement factor EF^{SERS} for SO in solution, in our experimental conditions, is ≈30000.

We followed the same approach when dealing with the powders prepared by drying drops of SO solution and colloidal dispersion on the glass slide. The drops dry on the glass slide leaving a 3.5 mm diameter circular deposit where the sample is dispersed. The laser spot is focused with a 50× microscope objective and it has about 2 μm diameter in the focus. The number of SO active in the SERS spectrum with respect to the total number of SO molecules present should be the same as in the solution experiments (if no change in the SO-AgNPs interaction occurs). Therefore, the SERS enhancement factor EF^{SERS} in the powder resulting from the drops deposited on the slide, evaluated on the major peaks in the Raman and SERS spectra shown in Fig. 3, is as large as 8000 ± 1000. We made no correction on the possible optical thickness of the sample as measurements were taken at the solid surface and the dye content in the two cases is very similar.

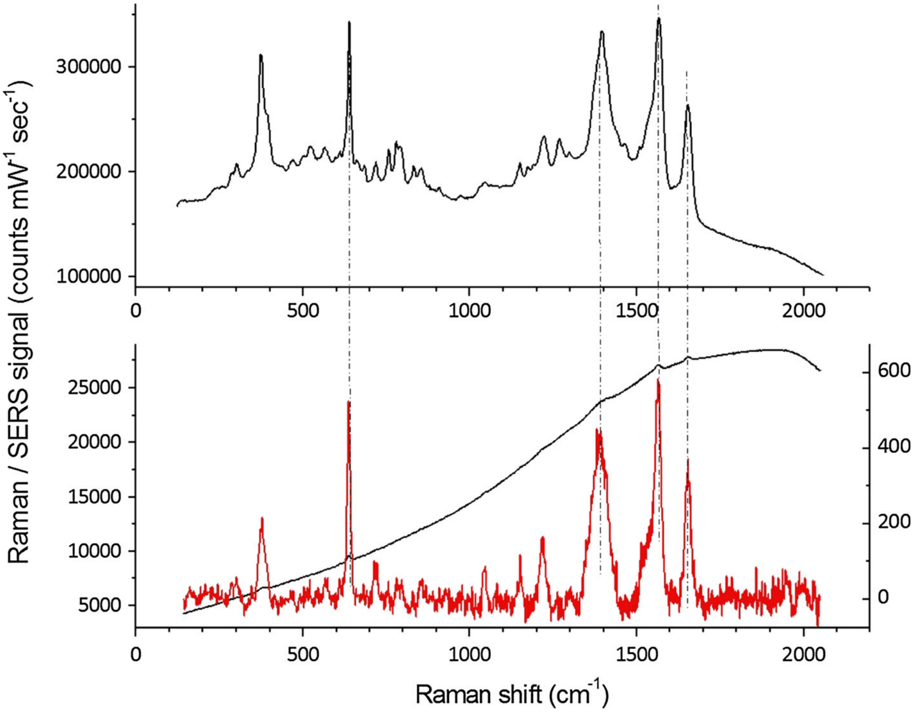

Fig. 3 The Raman spectrum of SO powder is shown in the *bottom panel*, both as raw data (*black trace*, left axis) and as background-subtracted data (*red trace*, right axis, units: counts mW^{-1} s^{-1}). The SERS spectrum (raw data) of SO powder in presence of AgNPs is shown in the upper panel. We used the four bands connected by vertical lines for the calculation of the SERS enhancement factors. Experimental conditions: excitation at 632.8 nm, laser power 0.7 mW at the sample, microscope objective ×50

Again, we followed the same approach when dealing with the dispersions of SO in the gel. The samples were prepared by cutting small agar–agar gel cubes with 5 mm side, adding a few drops of the appropriate solution on top of the cubes, and letting the cubes to dry in air before undertaking the Raman/SERS measurements. The dried samples appear uniform in the top and no single grains of dye are present. In the case of the water-based agar gel doped with equal volumes of the colloidal dispersion and the dye solution, the percentage of SO molecules active in the SERS spectrum should be the same as for the other samples (if our adsorption model for SO on the AgNPs holds also for this system). Definitely different is the case of hydrogels preloaded with AgNPs: here we used the colloidal solution (in place of water) in the hydrogel preparation and after we add on top of the bead the usual 4 μL of the AgNPs colloidal dispersion and 4 μL of 10^{-4} M SO solution in water. Thus, given that the density of the agar gel equals that of the colloidal solution, we have 129 μL of colloidal solution (1.0×10^{-9} M in AgNPs) per 4 μL (1.0×10^{-4} M in SO) of dye solution. It corresponds to a numerical ratio of ~3000 between the number of SO molecules and the AgNPs. Therefore, according to our adsorption model, almost all the SO molecules are participating to the SERS signal. We report in Fig. 4 the Raman spectra of the different samples made out of the agar hydrogels. In the bottom panel we show the Raman spectrum (raw and background subtracted data) obtained from a gel doped with the 10^{-2} M SO water solution. In the other panels we report the SERS spectra

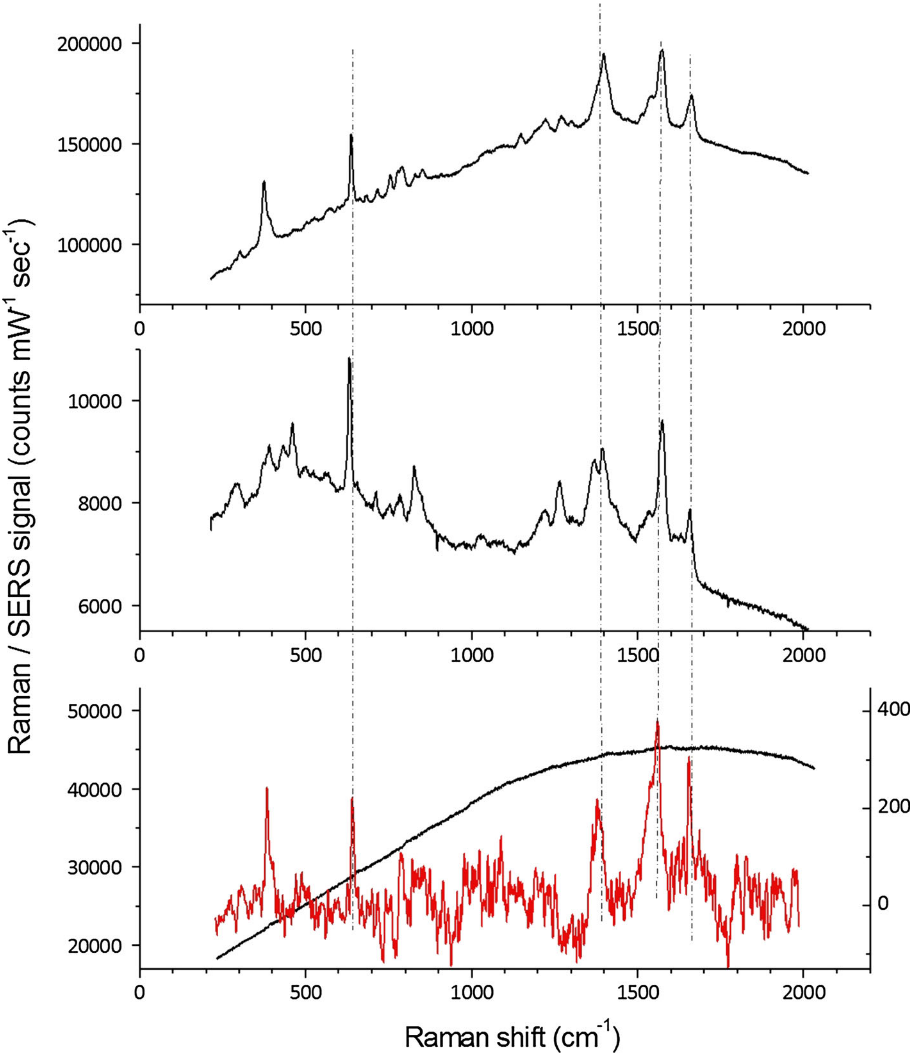

Fig. 4 The Raman spectrum of SO dispersed in the agar gel is shown in the *bottom panel*, both as raw data (*black trace*, left axis) and as background-subtracted data (*red trace*, right axis, units: counts mW^{-1} s^{-1}). The SERS spectrum (raw data) of SO dispersed in the water based gel and in the AgNPs loaded agar gel are shown in the *middle and upper panels*, respectively. We used the four bands connected by *vertical lines* for the calculation of the SERS enhancement factors. Experimental conditions: excitation at 632.8 nm, laser power 0.7 mW at the sample, microscope objective ×50

measured on gels doped with the 1:1 mixture of the 10^{-4} M SO water solution and 10^{-9} M AgNPs colloidal dispersion: the result for the water-based hydrogel are shown in the middle panel, and those for the colloidal dispersion based hydrogel are shown in the top panel.

Then, the SERS enhancement factor EF^{SERS} in the gel, evaluated on the major peaks in the Raman and SERS spectra shown in Fig. 3, is as large as 28500 ± 3000 for the water-based gel and 30000 ± 3000 for the AgNPs-preloaded gel. We made no correction on the possible optical thickness of the sample as measurements were taken at the solid surface under tight focusing conditions.

All the different determinations of the SERS enhancement factor for the solution, powder, and gels are of the same order of magnitude (10^4). This strongly support our assumption that the dye-AgNPs interaction occurs with very similar mechanisms in the different systems we studied. Under this respect, the very close enhancement factors determined for small and large amounts of AgNPs in the hydrogel with respect to the amount of dye provide a convincing demonstration of the effectiveness of our model. A smaller value for the enhancement factor is determined for the solid samples. However, we must notice that this is the ill-defined system as, in this case, we noticed the most inhomogeneous distribution of materials in the sample and, probably, a larger statistics cloud have been useful. A second general comment we can make on this result is that the SERS amplification does not increase with the density of NPs that occurs in going from the solution to the gel or solid phases. Thus, the speculation on the possible creation of “hot spots”, due to the closeness of the NPs in the dry sample upon the shrinking process, was not correct.

4 Conclusions

We provided an assessment of the relative SERS enhancement factor for the Raman spectrum of a cationic dye in presence of AgNPs, either in a colloidal dispersion, in a hydrogel or powders. The resulting enhancement factors are determined, through a simple model, in the order of magnitude of 10^4.

This work did try to establish, also, the reasons behind the very good efficiency we observed for the SERS identification of dyes after their extraction by an agar hydrogel. Our present results clearly demonstrate that the key factor for its high sensitivity is the concentration effect occurring by the shrinking of the gel upon drying. The height of the gel bead shrinks from a few millimeter size (typically 5 mm) to a sub-millimeter size, instead the base area reduction is much smaller. This corresponds to an overall volume contraction by at least one order of magnitude and therefore an effective concentration of the analyte in the extracting medium before the measurements, which are taken on small portions of the sample surface.

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