Surface-Enhanced Raman Scattering Spectral Imaging for the Attomolar Range Detection of Crystal Violet in Contaminated Water

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ABSTRACT: A series of nanocomposites based on polyamide (NL16, PA) filter membranes containing metal nanoparticles (NPs) have been prepared by filtration under reduced pressure of the metal colloids. The ensuing materials were then investigated as substrates for surface-enhanced Raman scattering (SERS) imaging studies envisaging the spectroscopic detection of vestigial organic pollutants dissolved in contaminated water. The organic dye crystal violet (CV) was used here as a model pollutant because it is a hazardous compound present in certain effluent waters. Moreover this compound is well-known for its strong SERS activity, which is clearly advantageous in the context of material development for SERS. Indeed, several preparative strategies were employed to prepare PA-based composites, and the impact on SERS detection was investigated. These include the use of chemical and morphological distinct plasmonic NPs (Ag, Au), a variable metal load and changing the order of addition of the analytical specimens. These studies demonstrate that the parameters employed in the fabrication of the SERS substrates have a strong impact on the Raman signal enhancement. The use of Raman imaging during the fabrication process allows establishing improvements that translate to better performances of the substrates in the analyte detection. The results have been interpreted by considering an integrated set of operational parameters that include the affinity of CV molecules to the substrate, amount and dispersion of NPs in the PA membranes, and the detection method. Noteworthy the use of SERS analysis assisted with Raman imaging allowed achieving a detection limit for CV as low as 100 aM in ultrapure water and 10 fM in real samples.

INTRODUCTION

The contamination of natural waters with organic dyes has been of general concern because these compounds are potentially harmful. In natural waters, such organic dyes can reduce sunlight penetration and deplete the dissolved oxygen. Also, mutagenic and carcinogenic intermediates can be produced by hydrolysis or oxidation of such organic dyes, with impact in aquatic organisms as well as animals and humans. Crystal violet (CV) is a synthetic basic cationic dye of the triarylmethane type that imparts a beautiful violet color to aqueous solutions. CV is widely used for dyeing textiles and used as a component of printing inks and paints. In addition, for many years, CV was used in veterinary medicine for the treatment of fungal infections in aquacultures; in consequence, vestigial traces of CV have been found in aquaculture products, namely, in fish. Currently, the presence of CV in food is not allowed in the European Union.

Environmental monitoring on a routine basis and in end user local points might mitigate pernicious effects, but there is need of effective and easy implementation methods to detect vestigial amounts of CV in effluent waters. In the past decades, analytical techniques based on the surface-enhanced Raman scattering (SERS) effect have emerged as an alternative strategy for the detection of specific water pollutants because of its relevant features, such as high sensitivity associated with unique spectroscopic fingerprints of selected compounds. SERS can also be regarded as a complementary tool to other techniques, namely, in analytical protocols employed in field analysis that use portable Raman equipment. Our own interest in this topic has been guided by the development of reproducible substrates that are sensitive to target analytes, which is a crucial aspect in SERS analysis.

Progress has been made for the fabrication of stable, low-cost, and active SERS substrates with large-area uniformity that allows reproducible signal Raman enhancements. Moreover, metal nanoparticles (NPs) assembled on paper or other flexible materials offer new possibilities for developing low-cost and facilely handled SERS sensors. An interesting advancement in the fabrication of filtering sensors has been reported by Yu and White, which used a straightforward method to fabricate handy SERS substrates composed of polyamide (PA) membranes and Ag NPs. Briefly, the colloidal NPs were retained in the PA membrane via syringe
filtration and then the sample solution was passed through the metal-loaded filter membrane. The same authors have demonstrated that the PA/Ag filters could detect low concentrations of rhodamine 6G (R6G), melamine, and malathion in water but without exploring Raman imaging. Here, we have prepared PA-based composites loaded with metal NPs by filtering under reduced pressure the respective colloids. These nanocomposites were prepared by using colloids (Ag, Au) having different particles’ morphologies (spheres and stars) and variable metal load. Using Raman imaging coupled with SERS analysis, our studies demonstrate that the variation of such parameters has a strong impact on the

Figure 1. Visible spectra and TEM images of the metal colloids: (a) Ag nanospheres (Ag NPs), (b) Au nanospheres with 10 nm average size (Au-10 NPs), (c) Au nanospheres with 30 nm average size (Au-30 NPs), and (d) Au nanostars (Au NSs).
Raman signal enhancement. Also, we wish to demonstrate that by employing both Raman imaging and SERS analysis, not only the substrate fabrication can be optimized but also lower detection limits can be achieved.

## RESULTS AND DISCUSSION

Aqueous solutions of CV have strong absorption in the visible because of a band peaked at 586 nm that extends to a spectral window that includes the excitation wavelength (532 nm) used for acquiring the Raman spectra (see the Supporting Information, Figure S1-1). The Raman spectrum of CV aqueous solution (0.1 M) is presented in Figure S1-2 (see the Supporting Information). Because the excitation wavelength is within the wavelength region of absorption for CV, there is a Raman resonance effect that can be explored together with the surface-enhanced Raman effect for detecting CV molecules close to plasmonic metal surfaces (surface-enhanced resonance Raman scattering).

The metal NPs used in this research have been prepared using distinct colloidal syntheses. The colloidal NPs employed in the preparation of PA-based composites were selected based on their facile loading into the PA membranes and because they are known to have good SERS activity. Figure 1 illustrates the visible spectra of the metal NPs with distinct nature and morphologies together with the corresponding transmission electron microscopy (TEM) images of the colloids. A solution of 20 mL divided by four portions of 5 mL (4 × 5 mL) of each colloid sample was passed through a PA membrane and dried at room temperature overnight, to obtain the respective NPs/PA substrate.

In the first stage, Raman imaging of the NPs/PA substrates was performed after dropping an aliquot (10 μL) of an aqueous solution of CV (100 μM), to assess the respective SERS response to CV. Figure 2 shows the average Raman spectra of CV (100 μM) in the NPs/PA substrates, obtained from the spectral data acquired in Raman imaging using the following samples as substrates: (a) Ag/PA; (b) Au-10/PA; (c) Au-30/PA; (d) Au NSs/PA; and (e) PA (excitation at 532 nm, 40 × 40 μm, 150 points per 150 lines, one acquisition with 0.05 s each point, 15 μW).

Figure 2. Average Raman spectra of CV (100 μM) obtained from the spectral data acquired in Raman imaging using the following samples as substrates: (a) Ag/PA; (b) Au-10/PA; (c) Au-30/PA; (d) Au NSs/PA; and (e) PA (excitation at 532 nm, 40 × 40 μm, 150 points per 150 lines, one acquisition with 0.05 s each point, 15 μW).

CV (100 μM) in the NPs/PA substrates, obtained from the spectral data acquired in Raman imaging. First, it is observed that the SERS spectra in Figure 2a–d are similar to the conventional Raman spectra of a CV aqueous solution (0.1 M, presented in Figure S1-2). Control experiments recording the Raman spectra of aqueous solutions with the same concentration in the CV analyte (100 μM) did not give a Raman signal. Also, the Raman spectra of neat PA membranes, that is, without metal NPs, with the addition of the same amount of CV, did not give Raman bands that could be ascribed to CV, in all areas analyzed over the PA substrate (Figure 2e).

These results demonstrate that there is a SERS effect for CV in the NPs/PA nanocomposites because of the presence of the metallic NPs incorporated within the PA matrices. For comparative purposes, the conventional Raman spectra of both PA filter membranes and Ag/PA composites are also presented in Figure S2 (see the Supporting Information). Moreover, the spectra were considered as representative after analyzing several areas of the substrate using Raman mapping and also by randomly selecting points on the substrate (10 000 μm × 10 000 μm) and recording the corresponding SERS spectra (Figure S3, Supporting Information). Although these results show slight changes in the Raman intensities, it is clear that at this scale the SERS signals are always observed regardless the region analyzed. Figure 2 also shows that under the conditions used, there are no significant differences in the SERS spectra of CV recorded with the different substrates.

The assignment of the CV Raman bands was based on literature reports including vibrational spectroscopic data for this organic dye.18–20 In brief, there are three different groups of vibrational modes; those associated with the central carbon atom (C–phenyl vibrations—up to 450 cm⁻¹), nitrogen atoms (N-phenyl stretching—between 1350 and 1400 cm⁻¹), and phenyl rings (skeletal ring vibrations and ring C–H deformations—between 400 and 1300 cm⁻¹ and ring stretching modes—above 1400 cm⁻¹). Raman bands were observed for C–phenyl bending at 437 cm⁻¹, ring C–H bending at 1171 cm⁻¹, ring skeletal vibration of radical orientation at 915 cm⁻¹, N-phenyl stretching at 1371 cm⁻¹, and ring C–C stretching at 1531 and 1620 cm⁻¹. The N-phenyl stretching band at 1371 cm⁻¹ and the ring C–C stretching band at 1620 cm⁻¹ were used to perform Raman imaging (obtained with the integrated intensity of the respective band), with the last one often used to calculate the enhancement factors in reported SERS studies.20,21 This result also indicates that the substrate containing Ag NPs presents the best SERS activity among the substrates tested; thus, in the subsequent experiments, the substrates loaded with Ag NPs have been preferentially used.

The commercial membranes used in this research are made of pure PA and normally employed as filters for water clarification and sterile filtration. These membranes are relatively cheap and mechanically robust during colloidal filtration, which make them suitable to fabricate the SERS substrates described here. The scanning electron microscopy (SEM) and atomic force microscopy (AFM) images presented in the supporting information (Figure S4) show that the PA membranes have a fibrous microstructure that result from a network of interlaced PA microfibers. The root mean square surface roughness of a typical PA membrane was 282 nm over 15 × 15 μm² area, as determined by AFM. The SEM images of the Ag-loaded PA samples (Figure 3a) show that the metal NPs can be supported by the fibrillar structure, thus providing regions with a high density of SERS-active NPs. The homogeneity of SERS-active solid substrates, in particular at the nanoscale, has been a challenging topic, which is crucial to implement such materials as analytical platforms.23–26 To assess the homogeneity of the metal-loaded PA substrates, the Raman image showing the spatial distribution of CV molecules (100 μM) on the Ag/PA membrane surfaces was acquired and is presented in Figure 3b for the best SERS operational conditions. High-resolution Raman imaging was performed.
using 22 500 spectra by raster-scanning the laser beam over a surface area of 40 × 40 μm, providing a spatial resolution of approximately 0.27 μm. The integration of the absolute area underneath the band at 1371 cm⁻¹ (N-phenyl stretching) was used to establish the color intensity and create the Raman image. Thus, the brighter colors in the image indicate regions with stronger SERS signal because of the presence of CV molecules adsorbed on the Ag NPs, simultaneously indicating the distribution of the Ag NPs over the membrane. The Raman image in Figure 3b shows that the Ag NPs are evenly dispersed over the surface of the PA membrane, which is in agreement with the SEM results discussed above.

To develop reproducible SERS substrates based on these PA nanocomposites, it is important to evaluate the in-depth dispersion of the metal NPs in the PA matrix. In this context, three-dimensional (3D) Raman images are of great interest because they provide information about the distribution of the Ag NPs throughout the analyzed substrate (surface and interior). Thus, 3D Raman images of the Ag–PA filter membrane (4 × 5 mL) probed with a CV aqueous solution (10 μL, 100 μM) were built by the acquisition of a series of two-dimensional (2D) images along the z-direction (down to −5 μm). The 2D Raman images with 2.5 μm apart in the z-direction are shown in Figure 4. The 2D images were then combined into a 3D image stack (Figure 4-bottom). The resulting 3D image provides information about the bulk distribution homogeneity of assemblies comprising Ag NPs and CV molecules in the PA filter membranes. The white arrows in Figure 4 show positions in the substrate where the Raman signal of CV was not observed at z = 0 μm (surface of the substrate) but where it was clearly detected down at z = −5 μm (in spots marked by green arrows, vertically aligned with the surface ones). This result confirms the homogeneous distribution of Ag NPs inside the PA matrix. During the filtration of the metal colloid under reduced pressure, a certain amount of Ag NPs are attached to the PA fibers at the surface but others migrate through the porous matrix and are retained within the interior of the PA membranes. Therefore, when a drop of CV is placed on the top of the substrate, the CV molecules (~1.4 nm) diffuse through the porous matrix and interact with the Ag NPs distributed inside the PA membrane. Results reported by Kudelski have shown that relatively large...
molecules, such as those of CV, can interact with SERS-active sites formed by rearrangement of the metal NPs (likely mechanism in metal sols) and also after diffusion toward already arranged SERS-active sites.25

The dark regions indicated by the white arrows in the 2D Raman image taken at \( z = 0 \ \mu m \) (surface) can be associated with the deformation of the membrane because of the filtration method under reduced pressure used for the substrate preparation. PA filter membranes with a porous microstructure of 800 nm (instead of the 200 nm used in this work) were also tested and gave the same type of dark regions in the Raman image. Using a similar preparation method, but with a PA filter membrane (200 nm) that is coated with Ag NPs without filtration under reduced pressure, the Raman images did not present those dark regions on the surface, and the Ag NPs can only be detected at the surface (Figure S5).

It has been reported that SERS activity in a solid substrate is highest in metal nanojunctions (hotspots), because of a strongly enhanced local electromagnetic field when irradiated with laser light.12,26−31 In principle, for the PA membranes investigated here, the formation of such hotspots can be tuned by adjusting the amount of metal NPs and aggregation state in the matrix. This can be simply assessed by varying the volume of the Ag colloid that passes through the PA filter membrane, in distinct filtering steps, keeping the total volume constant. Thus, let us consider a total volume of 20 mL in the Ag colloid, used in each of the following filtration procedures (under reduced pressure): \( 1 \times 20, 2 \times 10 \) and \( 4 \times 5 \) mL. Figure 5 shows the SERS images of the spatial distribution of CV molecules in different Ag/PA membranes, prepared as described above, after dropping an aliquot (10 \( \mu L \)) of an aqueous solution of CV (100 \( \mu M \)). Raman images were generated using the intensity of the Raman band at 1371 cm\(^{-1}\) from 22,500 spectra. The brighter yellow areas at the surface of the substrate, corresponding to the strongest SERS signal of CV, decreased from the Ag/PA substrate obtained by filtering the Ag colloid in a single step (\( 1 \times 20 \) mL) to the Ag/PA substrate obtained after multistep filtration. The results shown previously in this paper were obtained with Ag/PA substrates prepared using a 4 \( \times 5 \) mL Ag colloid filtration (Figure 5c corresponds to Figure 3, presenting a different color intensity scale).

Figure 6a,b shows the 2D Raman images along the z-direction (down to −5 \( \mu m \)) for both substrates prepared by filtration of Ag NPs in \( 1 \times 20 \) and \( 2 \times 10 \) mL steps, respectively. For the substrate obtained after filtration in a single step, the SERS signal of CV is strongest at the surface of the substrate (\( z = 0 \ \mu m \)) (Figure 6a), decreasing in intensity as the SERS analysis is performed in depth (along z).

The tendency shown in Figure 6a, and less in Figure 6b, of decreasing of the SERS signal of CV along the z-direction, is not observed for the Ag-loaded PA membranes obtained in multistep filtrations. A plausible explanation for such differences relates to the influence of the filtration method applied to the colloid on the aggregation state of the Ag NPs entrapped in the PA membranes. A single step filtration under reduced pressure induces the formation of Ag aggregates mostly at the solid/liquid interface over the membrane, whereas the same amount of colloidal Ag NPs will be better distributed within the PA bulk matrix when the same volume of Ag colloid is divided in smaller portions for multistep filtration. It should be noticed that this behavior was also observed for Ag/PA prepared with 4 \( \times 5 \) mL (Figure 4), in which a similar SERS intensity of CV was observed along the z-direction, indicating a better distribution of the Ag NPs inside the PA filter membrane.
This result was also confirmed by SEM, where Ag NP aggregates are observed for the substrate using a single step filtration, and a better distribution of the Ag NPs is shown on the substrate in which the volume of the Ag colloid was divided two times (Figure S6, Supporting Information). To have additional evidence for the dependence of the SERS activity on the aggregation state of the Ag NPs in the composite membranes, distinct total volumes of the Ag colloid have been employed in their fabrication. Thus, Ag colloid was passed through the PA filter membranes in single steps, using the following volumes in each: 20, 10, 5, and 2 mL. The as-prepared Ag-loaded PA membranes were then evaluated as SERS substrates, after the addition of CV solution (100 μM, 10 μL), by monitoring the band of CV at 1371 cm⁻¹ to generate a Raman image (Figure 7). The plot of the average intensity of the N-phenyl stretching mode at 1371 cm⁻¹ and the C–C stretching of the ring at 1620 cm⁻¹ are also presented in Figure 7c, for the respective substrates. The SERS intensity for CV decreased from the Ag/PA substrate obtained using 20 mL of the Ag colloid to the Ag/PA substrate obtained by using the smallest volume of 2 mL. It should be highlighted that the SERS intensity for CV using the Ag/PA substrate with 20 mL of Ag colloid is slightly higher than the Ag/PA substrate with 10 mL of Ag colloid; thus, in the subsequent experiments, the substrates loaded with 10 mL of Ag colloid were used, which is a compromise between SERS intensity and the Ag colloid used.

A great advantage in using the PA membrane-based SERS substrates is the ability to preconcentrate the analyte under study, similarly to what happen in a solid-phase extraction method. The platforms presented here combine two important assets for chemical analysis, the fast collection of the specimen and the in situ detection, in this case by SERS. To collect the analyte, two simple procedures can be employed, either by filtering the sample solution or by just dropping some aliquots on the top of the membrane. Figure 8a,b illustrates the results when the SERS performance of the filter membranes was evaluated using both methods for sample preparation. Hence, the Raman signal intensity was far better by filtration the sample solution or by just dropping 10 mL of CV (aqueous solution 100 μM) than by dropping 10 μL of solution with the same concentration over the membrane, which is line with a better diffusion and extraction of the analyte within the matrix. Figure 8c also shows the SERS spectrum of CV retained in the PA filter membranes prior to loading of the colloidal Ag NPs (10 mL). In the latter case, less CV molecules were retained in the PA membrane and available for adsorption in the Ag NPs deposited afterward. Although the latter method is less SERS effective, a detection limit for CV as low as 1 nM was still reached. The focus here was to evaluate this method to detect organic dyes in water, by selecting CV as a pollutant model because of their relevance as potential contaminants in industrial effluents. Following this objective, two other organic dyes were preliminarily analyzed in similar conditions as those employed in the analysis of CV. Figure S7 shows that good SERS spectra were also obtained for methylene blue (MB) and R6G (100 μM concentration) by using the Ag/PA filter membranes as substrates.

The usefulness of the Ag/PA filter membranes was also evaluated as SERS platforms for monitoring vestigial CV in more complex matrices. Thus, Ag/PA filter membranes (prepared by filtration of 10 mL of Ag colloid in one step) were used in the extraction and SERS detection of CV previously dissolved (at 100 μM concentration) in seawater collected from Aveiro Estuary. Figure 9 presents the Raman images obtained with the integrated intensity of the characteristic band of CV at 1370 cm⁻¹ using Ag/PA as SERS substrate for samples of CV-spiked Aveiro Estuary water. The SERS images show the spatial distribution of CV molecules on the Ag/PA substrate, using different CV concentrations. The detection limit of CV in the Aveiro estuary water samples was 10 fM. The Raman spectra obtained for the detection limits determination are presented in Figure 9d, and all characteristic Raman bands of CV were clearly observed in such diluted samples. Plots of the intensity of the 1370 and 1620 cm⁻¹ CV Raman bands for various CV concentrations are also presented in Figure 9e.

It should be highlighted that such lower SERS detection limits for CV molecules could only be achieved using Raman imaging coupled with SERS. Figure 9c shows the Raman image of the 1371 cm⁻¹ vibrational mode collected from the Ag/PA filter membrane with the lowest CV concentration (10 fM) on
Aveiro Estuary water. The Raman image demonstrates that the SERS signal of CV is highly localized, corresponding only to a few active Raman scattering sites on the Ag/PA filter membrane. The brighter areas observed in Figure 9c clearly reveal that these hotspots promote a higher enhancement of the CV Raman signal, making possible the detection of CV molecules even from such a low analyte concentration. The SERS spectra in Figure 9d are single Raman spectra taken from the brightness spots observed in the respective Raman images (1 acquisition, 0.05 s).

For the same experiment but using ultrapure water, the achieved detection limit was 100 aM (Figure S8). This result demonstrates that the Ag/PA filter membrane can be used as a SERS substrate for CV detection in real samples, is not limited to SERS analysis in ultrapure water CV solutions, and show extreme SERS sensitivity and good reproducibility.

**CONCLUSION**

In conclusion, we have demonstrated the potential of Raman imaging methods to monitor the fabrication process of SERS substrates based on PA filter membranes and loaded metal colloidal NPs. Raman imaging coupled with SERS allows for adjusting operational parameters such as the amount and dispersion of NPs in the PA membranes, the sample preparation method, and also for monitoring the formation of active SERS sites in distinct regions of the membranes. Because
of the increasing number of technologies requiring flexible SERS substrates, this approach might give a new impetus for a better understanding of how the Ag NPs incorporated in the matrix affect the SERS performance of the substrates.

A number of advantages can be identified for the use of these platforms instead of conventional SERS substrates, namely, their flexibility, efficient extraction of chemical compounds, low cost, and high surface area, making these filter membranes excellent platforms for trace detection of chemicals and water pollutants. In addition, the fabrication process demonstrated here can be easily scaled up and large amounts of contaminated water can be used for in field measurements.

■ EXPERIMENTAL SECTION

PA filter membranes (NL16, PA), 47 mm diameter, 0.2 μm pore sizes, were purchased from GE Healthcare Life Science (Whatman) for performing the filter SERS assay. The following chemicals were used as purchased: hydrogen tetrachloroaurate (III) trihydrate (HAuCl4·3H2O, 99.9%, Sigma-Aldrich), silver nitrate (AgNO3, 99.9%, J. M. Vaz Pereira), trisodium citrate dihydrate (Na3C6H5O7·2H2O, 99%, Sigma-Aldrich), CV (C25H30ClN3, Merck), polyvinylpyrrolidone (PVP, wt 10 000, Sigma-Aldrich), ethanol (C2H5OH, absolute anhydrous, Carlos Erba) and N,N-dimethylformamide (DMF, Carlos Erba), MB (C16H18N3SCl, Riedel-De Haën), and R6G (C28H31N2O3Cl, Sigma-Aldrich).

Figure 8. SERS spectra of CV upon (a) filtration under reduced pressure of CV aqueous solution 100 μM), (b) drop and evaporation of the same CV solution, and (c) addition of the CV solution before adding the Ag NPs by filtration.

Figure 9. Raman images obtained with the integrated intensity of the band at 1371 cm⁻¹ of CV recorded using Ag/PA membranes as the SERS substrate for samples of CV spiked on Aveiro Estuary water using different CV concentrations: (a) 1 μM; (b) 10 nM; and (c) 10 fM. The vertical bar shows the color profile in each image, with the relative intensity scale. d) Single SERS spectra of CV taken from the Raman images in Aveiro Estuary water with several CV concentrations; (e) Plots of the intensity of the 1371 and 1620 cm⁻¹ CV Raman bands for various CV concentrations, using five different samples (Ag/PA) for each concentration.
Silver spherical NPs: The Ag colloids were prepared by reduction of aqueous AgNO₃ with sodium citrate. An aqueous solution (400 mL) of AgNO₃ (1 mM) was boiled under reflux (90 °C). Then, a sodium citrate solution (100 mg) was added dropwise to the boiling silver nitrate solution, under vigorous stirring. The mixture was then refluxed for more than 10 min and then cooled at room temperature.

Gold spherical NPs: The Au colloid with 10 nm diameter was prepared via reduction of a gold(III) salt with trisodium citrate as follows. An aqueous solution containing 10 mL trisodium citrate (38.8 mM) was mixed with 100 mL of tetrachlorauric(III) acid solution (1 mM) previously brought to a rolling boil under vigorous stirring. The colloidal Au nanospheres were placed in a bottle, and this was kept in the dark at a temperature of 5 °C. Gold NPs with 30 nm were also prepared via reduction of a gold(III) salt with trisodium citrate, using different amounts of HAuCl₄ (200 mL, 0.3 mM) and sodium citrate (1.8 mL, 38.8 mM).

Gold nanostars: The capping of the as-prepared Au NPs with 10 nm diameter was modified with PVP by the method elsewhere and then used as seeds for nanostar growth. Nanostars were prepared by adding PVP-coated gold seeds ([Au]: 9.29 × 10⁻⁴ M) in ethanol to a mixture of HAuCl₄ (2.73 × 10⁻⁴ M) and PVP (monomer concentration: 10 mM) in DMF under rapid stirring at room temperature. Within 20 min, the color of the solution changed from pink to blue, indicating the formation of gold nanostars.

To perform the SERS filter membranes, we used two different methods: in the first method, the PA filter membrane was placed into a filter Millipore holder and then the plasmonic nanostructures were passed through the filter membrane by filtration under reduced pressure. In the case of Ag NPs, two filtering steps were used for distinct amounts of colloids. For instance, for the same initial volume of the Ag colloid added to the PA filter membrane, the Ag NPs were filtered (under reduced pressure), using the following procedure: 1 × 20, 2 × 10, and 4 × 5 mL. In a second approach, the total volume of the Ag colloid employed was also varied. Thus, PA composites were prepared by one-step filtration of the colloids by using the following volumes: 20, 10, 5, and 2 mL.

The membrane traps the NPs, forming a SERS-active substrate. The PA filter membranes loaded with NPs were then removed from the filter Millipore and dried at room temperature. The filter assays were investigated as SERS substrates for the detection of CV using different procedures: preconcentration by filtration under reduced pressure method and drop/evaporation, respectively. In the former, an aqueous solution of CV with 100 μM concentration has been prepared and 10 mL of the solution was filtered through the Ag/PA substrate. For the latter procedure, an aliquot of the aqueous solution of CV (100 μM, 10 μL) was added to the different metal/PA substrates and let to dry at room temperature.

In the last method, an aqueous solution of CV (10 mL, 100 μM) was passed through the filter membrane contained in the filter Millipore holder. After being dried at room temperature, the CV/PA filter membranes were placed again in the filter holder and then the colloidal Ag NPs (1 × 10 mL) were passed through the filter membrane. The composites were dried at room temperature.

For the detection of CV in real samples spiked with CV, a solution of CV (0.1 mM) was prepared in Aveiro Estuary water (seawater) and diluted from 100 μM to 100 aM, by sequential dilutions of a factor of 100, followed by filtration under reduced pressure. For the experiments with ultrapure water, aqueous solutions of CV with varying concentrations (from 100 μM to 1 aM) have been also prepared to establish the lower detection limit concentration. For all SERS measurements, the pure PA filter membrane was also used as the control sample. SERS measurements have been performed in different areas of the nanocomposites to check the reproducibility of the measurements.

Raman images obtained using the WITec alpha300 RA+ were produced by taking 150 × 150 Raman spectra in a uniform 40 × 40 μm² grid. A laser wavelength of 532 nm with a laser power of 15 μW was used. A 100X objective was used to view samples, and the integration time for each spectrum was 0.05 s. The times required to create Raman images using each integration time were 22 min. The plot for Raman intensity in relation to the CV concentration was generated by monitoring the band at 1371 cm⁻¹, assigned to the N-phenyl stretching mode and at 1620 cm⁻¹, assigned to the ring C–C stretching mode, using five different samples for Aveiro Estuary water.

A as-prepared Ag/PA composite was produced by taking 150 Raman spectra in a uniform 40 × 40 μm² grid. A laser wavelength of 532 nm with a laser power of 15 μW was used. A 100X objective was used to view samples, and the integration time for each spectrum was 0.05 s. The times required to create Raman images using each integration time were 22 min. The plot for Raman intensity in relation to the CV concentration was generated by monitoring the band at 1371 cm⁻¹, assigned to the N-phenyl stretching mode and at 1620 cm⁻¹, assigned to the ring C–C stretching mode, using five different samples for Aveiro Estuary water. The average Raman spectra were obtained for the average of all data taken to create the SERS images (22 500 spectra for each image). AFM in tapping mode (AC-AFM) was carried out using a tip-cantilever silicon reflex-coated with a spring constant of k = 42 N/m and 285 kHz of resonance frequency.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b01983.

Optical spectrum of CV; conventional Raman spectrum of CV aqueous solution, solid Ag/PA and PA filter; SERS spectra of CV obtained on different points of the Ag/PA substrate; SEM image of PA and AFM topography image; in-depth SERS images of CV in Ag/PA substrates; SERS spectra of MB; and R6G using Ag/PA filter membrane and SERS images in Ag/PA for different CV concentrations in ultrapure water (PDF).

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ACKNOWLEDGMENTS

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, POCI-01-0145-FEDER-007679 (FCT Ref. UID/CTM/50011/2013), financed by national funds through the FCT/MEC and when appropriate co-financed by Fundo Europeu de Desenvolvimento Regional (FEDER) under the PT2020 Partnership Agreement. S.F. thanks Fundação para a Ciência e Tecnologia (FCT) for the grant SFRH/BPD/93547/2013.

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