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ABSTRACT

Surface enhanced Raman scattering (SERS) is a powerful technique based on the intensification of the Raman signal because of the interaction of a molecule with a nanostructured metal surface. Electrochemically roughened silver has been widely used as SERS substrate in the qualitative detection of analytes at the ultra-trace level. However, its potential for quantitative analysis has not been widely exploited yet. In this work, the combination of time-resolved Raman spectroelectrochemistry with silver screen-printed electrodes (SPE) is proposed as a novel methodology for the preparation of SERS substrates. The *in situ* activation of a SERS substrate is performed simultaneously with the analytical detection of a probe molecule, controlling the process related to the preparation of the substrate and performing the analytical measurement in real time. The results show the good performance of silver SPE as electrochemically-induced surface-enhanced Raman scattering substrates. Raman spectra were recorded at fairly low integration times (250 ms), obtaining useful spectroelectrochemical information of the processes occurring at the SPE surface with excellent time-resolution. By recording the microscopic surface images at different times during the experiment, we correlated the different data obtained: structural, optical and electrochemical. Finally, the *in situ* activation process was used to obtain a suitable *in situ* SERS signal for ferricyanide and tris(bipyridine)ruthenium(II) quantification. The detection of the analytes at concentrations of a few tens of nM was possible with a low integration time (2 s) and good precision, demonstrating the exceptional performance of the Raman spectroelectrochemical method and the possibility to use cost-effective screen-printed electrodes for applications where a high sensitivity is needed.

KEYWORDS: surface-enhanced Raman scattering; screen-printed electrodes; electrochemistry; Raman spectroelectrochemistry

INTRODUCTION

Surface enhanced Raman scattering (SERS) was first introduced by Fleischman *et al.* in 1974, from their studies using electrochemically roughened silver surfaces and pyridine as a model Raman probe [1]. In this experiment was put in evidence the spectacular increment of the Raman signal in a roughened silver electrode. Thenceforth, the use of such SERS substrates was generalized and extended to other metals such as gold and copper [2]. Since the first discovery, SERS substrates have been mainly used for qualitative analysis [3–6]. However, the quantitative approach has not been widely exploited yet, motivated by some problems regarding the sensitivity and/or the reproducibility, among others. In this sense, the recent use of metal nanoparticles (NPs), which provide a better SERS signal, have displaced the employment of silver roughened substrates in real applications, mainly due to the highest sensitivity achieved [7,8].

Due to the importance of Raman spectroscopy as a characterization technique, when it is combined with powerful quantitative electrochemical methods, the potential of the technique is significantly amplified [9,10]. Raman spectroelectrochemistry (Raman SEC) provides information about the vibrational states of molecules, and therefore, about their functional groups and structure, which makes it one of the most useful spectroelectrochemical techniques [11,12]. Its combination with an easy and rapid substrate preparation elevates it to a top interest in the field of chemical analysis, especially for traces and ultratraces, taking advantage of the high sensitivity achieved by the SERS effect. Additionally, if the spectroelectrochemical experiment is carried out resolved in time (TR-SEC), the possibilities to obtain proper results are improved due to the careful control in the potential applied (dynamic electrochemistry) [13], which set a difference with the application of discrete potential values (stationary electrochemistry) [14,15]. TR- SEC experiments open the possibility of controlling the electrochemical potential of the system in a precise way and at any time of the experiment, which is a plus for SEC. Thus, TR-SEC allows, using

most of the potentialities of the dynamic electrochemical techniques, opening a wide spectrum of new possibilities such as the characterization of materials in operando conditions [16].

In spite of the current generalized use of metal NPs in many quantitative electrochemical surface enhanced Raman scattering (EC-SERS) experiments [10,14], the employment of roughened metal electrodes presents some advantages over substrates based on chemically synthesized NPs, because the preparation is easier, faster and simpler (it does not require complicated experimental protocols) and could offer *in situ* activation [17].

Therefore, the use of an electrochemical pretreatment to achieve a roughened SERS substrate represents a benefit in this sense [1,18,19]. However, in most of the cases, due to the difficulty of coupling electrochemical techniques with Raman spectroscopy, the substrate preparation is carried out *ex situ*, and then, the species are left to adsorb on the surface for several minutes to obtain an appropriate optical signal [20,21]. Several examples of *ex situ* activated silver electrodes for SERS-based assays have been reported [22,23]. Although this approach is usually useful, a possible time-dependent decrease of the surface and plasmon properties of the substrate could occur (by adsorption of other species or changes in the surface features, etc.), which would lead to lower optical signals than the ones obtained by using *in situ* activation. Due to these unavoidable changes, many quantitative experiments carried out so far can suffer from lack of reproducibility, making the technique less reliable.

In order to overcome these problems, the *in situ* preparation of the SERS substrate provides, among others, the following advantages: 1) An accurate control of the electrochemical parameters such as oxidation and reduction potentials, as well as the scan rate or the pulsed time; 2) A careful control of the nanostructures prepared, due to the real and simultaneous monitoring of both electrochemical and spectroscopic signal; 3) The possibility of having the probe molecule while the substrate preparation is taking place, which could yield some spectroscopic signal with a much more analytical quality, obtaining

information about the SERS effect of the nanostructures formed on the electrode surface during all the experiment. These benefits in the simultaneous preparation and measurement process open a new avenue for analytical application of TR- Raman SEC taking advantage of the SERS effect.

On the other hand, screen-printed electrodes (SPEs) have recently been described as substrates for SERS [24,25] and there are only few examples where they are used as EC-SERS tools [26–28]. Among others, metal SPEs have the advantage of being disposable, cheap and easy-to-use electrodes. They also can be reproduced in shape and composition with a reasonable precision. Besides, all three electrodes are integrated in the same device, namely, position of the reference and counter electrode is absolutely reproducible, which could confer more repeatability in the protocol of preparation. Finally, SPEs could be coupled to a specific cell that provides a reproducible methodology in the experimental protocol. All these features make metal SPEs suitable candidates to be used in Raman SEC analytical application. Therefore, it is important to obtain information about the behavior of roughened Ag SPEs as *in situ* SERS substrates, and especially about the dynamic processes leading to the *in situ* activation. Furthermore, new uses of SPE as SERS substrates for the determination of certain analytes in an easy, rapid and cost-effective way can be a very promising for quantitative applications of TR- Raman SEC.

In the present work, the real-time *in situ* activation of readily-available silver SPEs with excellent time/potential resolution (one spectra acquired each 250 ms) is proposed to obtain information about the processes leading to a SERS active substrate. Scanning electron microscopy (SEM) was used to obtain information about the structural features of the silver SPEs at different stages of the substrate preparation, and correlate it with the Raman SEC results. Two common analytes were selected to show the capabilities of this methodology: ferricyanide and the ruthenium-bipyridine complex ($[\text{Ru}(\text{bpy})_3]^{2+}$). On the one hand, ferricyanide was used as proof of concept because cyanide-based species usually have vibrations that show few characteristic bands at frequencies above 2000 cm^{-1} , which are very useful for studying the SERS effect on screen-printed electrodes. For instance, the SERS effect of KCN was used

to study the electrodeposition of silver nanoparticles on platinum electrodes [29]. Ferri/ferrocyanide couple was also used as probe species for testing a simultaneous Raman and UV/VIS SEC cell [30]. It has also been observed that both the ferri/ferrocyanide strongly interact with Ag and Cu surfaces [31] by forming chemisorbed layers on the electrode surface. On the other hand, $[\text{Ru}(\text{bpy})_3]^{2+}$ is a very interesting molecule because it is widely employed as a model for SEC systems [32,33] and it is the most common detection label in electrochemiluminescence assays [34] due to its great performance and sensitivity. Some works dealing with the SERS detection of $[\text{Ru}(\text{bpy})_3]^{2+}$ using silver electrodes have been reported [35,36]. However, the SERS detection has been typically performed in a subsequent step after the electrochemical activation of the silver electrode. The objective of this work is to demonstrate the analytical capabilities of the *in situ* activated silver SPEs for the quantitative and precise detection of ferricyanide and $[\text{Ru}(\text{bpy})_3]^{2+}$ at very low concentrations using a fast and cost-effective methodology.

MATERIALS AND METHODS

Instrumentation

In situ Raman SEC was performed by using a compact and integrated instrument, SPELEC RAMAN (DropSens), which contains a laser source of 785 nm. This instrument was connected to a bifurcated reflection probe (DRP-RAMANPROBE) and a specific cell for screen-printed electrodes (DRP-RAMANCELL). The laser spot size is about 200 μm . SPELEC RAMAN was controlled by DropView SPELEC software, which allows performing simultaneous and real-time spectroelectrochemical experiments, with totally synchronized data acquisition. **Figure S1** shows the instrumental setup for Raman spectroelectrochemistry employed in this work. SPEs (DRP-C013, DropSens) were also used throughout the work. These devices consist of a flat ceramic card on which a three-electrode system comprising the electrochemical cell is screen-printed. The working silver electrode is circular with a diameter of 1.6 mm, and the device has also an auxiliary electrode made of carbon and a silver electrode which acts as a

pseudoreference. Carbon SPEs (DRP-110, DropSens) were also used. These devices have a 4 mm circular working electrode made of carbon, being the counter and reference the same as in the DRP-C013 electrodes. All spectroelectrochemical measurements were performed at room temperature and using a solution of 60 μ L. The reported potentials are related to the silver pseudoreference electrode.

A JEOL 6610LV SEM was used to characterize the working silver electrodes at different stages of the electrochemical activation. An open source python script developed by D. Martín-Yerga, particleCounter [37], was used for the automatic counting and sizing of particles from the SEM images.

Reagents and solutions

Potassium ferrocyanide, potassium ferricyanide, potassium chloride and tris(2,2'-bipyridyl)dichlororuthenium(II) hexahydrate $[\text{Ru}(\text{bpy})_3]^{2+}$ were purchased from Sigma. Ultrapure water obtained with a Millipore DirectQ purification system from Millipore was used throughout this work.

Raman SEC measurements

RamanSEC experiments for ferri/ferrocyanide electrochemical conversion using carbon electrodes were performed by applying a cyclic voltammetry from +0.50 to -0.40 V at a scan rate of 0.05 V s⁻¹, a step potential of 0.002 V. Raman spectra were registered with an integration time of 1 s and a laser power of 185 mW (considering the spot size, the power by surface area is around 65 W/cm²). SERS experiments with silver SPEs were performed by applying a linear sweep voltammetry technique from +0.30 V to -0.40 V at a scan rate of 0.05 V s⁻¹, a step potential of 0.002 V and equilibration time of 1 s. Raman spectra were registered with an integration time of 0.250 s and a laser power of 258 mW (power by surface area around 90 W/cm²). For quantitative measurements, the Raman integration time was increased to 2 s to be able to detect a higher number of photons, and, therefore, increase the sensitivity. The SERS signal depends on several experimental parameters, but the conditions were selected to generate a high intensity SERS effect.

RESULTS AND DISCUSSION

Time-Resolved Raman SEC of ferri/ferrocyanide on carbon SPEs

In order to demonstrate the correct performance of the Raman SEC instrument and the usefulness of TR-Raman SEC, the experimental setup was initially validated by following the electrochemical processes of the ferri/ferrocyanide redox couple on carbon SPEs. Both species can be easily differentiated by Raman detection since ferricyanide shows one typical band at 2125 cm^{-1} , whereas ferrocyanide shows two bands at 2049 and 2086 cm^{-1} (**Figure S2**). Although ferricyanide theoretically has two Raman vibrational bands in this range[38,39], they are very close in wavenumber, and, therefore, their resolution is difficult to achieve experimentally, resulting in a spectrum with one band. SEC measurements of 0.05 M ferricyanide in 0.1 M KCl were performed by recording a cyclic voltammetry between $+0.50\text{ V}$ to -0.40 V at a scan rate of 0.05 V s^{-1} . Raman spectra were acquired every 1 s , obtaining one spectra each 0.05 V , which is a fairly good temporal resolution. **Figure 1** shows a 3D-surface of the temporal evolution of the Raman spectra in the range $2000\text{-}2200\text{ cm}^{-1}$ (where the cyanide vibrational bands usually are observed) while performing a whole cyclic voltammetry. **Video S1** shows dynamically the evolution of the Raman spectra during the cyclic voltammetry, which clearly shows the correlation between the Raman response and the electrochemical transformation of the redox couple. The initial single band of ferricyanide at 2125 cm^{-1} decreased when the potential was scanned towards negative potentials. Then, when the electrochemical reaction takes place on the electrode surface and the ferricyanide is reduced, the two ferrocyanide Raman bands started to grow. The opposite spectroscopic process occurs when the potential was scanned in the reverse direction. This **Video S1** shows the excellent performance of the Raman SEC setup to study electrochemical processes that take place on the surface of SPE, and

highlights the time resolution of the technique which makes possible to study a dynamic electrochemical process.

[FIGURE 1]

Time-resolved dynamic study of the *in situ* EC-SERS effect on silver SPEs

After demonstrating the usefulness of the experimental setup in the study of dynamic electrochemical processes, the *in situ* electrochemical activation to generate a SERS silver SPE substrate was evaluated by TR-SERS SEC and microscopic imaging. After testing different oxidation-reduction potentials, scan rates and pretreatment times, some optimum conditions with high reproducibility and sensitivity were chosen. Again, ferri/ferrocyanide redox couple was chosen as a model electrochemical and Raman active system. A linear sweep voltammetry of ferricyanide 10^{-5} M in 0.1 M KCl from +0.30 V to -0.40 V at a scan rate of 0.05 V s^{-1} was performed. The SERS effect on the electrode surface was generated *in situ*, in order to study the process dynamically. Raman spectra were recorded simultaneously every 0.250 s, thus providing time-resolved information every 0.0125 V (it would be possible to use shorter integration times or slow scan rates if a higher SEC time resolution were necessary). Most Raman SEC studies have used higher integration times but only a few works have shown the usefulness of millisecond spectra acquisition [9,10]. This highlights one of the advantages of this technique, which allows us to get some specific Raman information at differential times. The electrochemical response obtained during the potentiodynamic experiment is illustrated in the **Figure 2A** together with the evolution of the peak intensity of the Raman band at 2078 cm^{-1} (band assigned to the cyanide-group vibrations), after a baseline correction. **Figure S3** shows the typical Raman spectra obtained at different potentials with the increased peak due to cyanide vibrations when the silver surface become SERS-active, whereas **Figures 2B-D** show the SEM images recorded when the linear sweep voltammetry is stopped at different potentials, in order to get structural information of the electrode surface during the SEC experiment. The volt-

ammetry starts with an intense anodic process indicating that a good amount of the silver surface is being oxidized at these potentials according to the reaction $\text{Ag}^0 \rightarrow \text{Ag(I)} + 1\text{e}^-$. In presence of Cl^- , insoluble AgCl is generated on the electrode surface, increasing the roughness and porosity in comparison to the pristine surface, as can be observed comparing the SEM images of the initial silver SPE (**Figure 2B**) and when the voltammetry was stopped at +0.05 V (**Figure 2C**). The initial homogeneous silver surface was transformed into a surface with numerous well-compacted submicron particles of AgCl, confirmed by EDX analysis and the appearance of a Raman band [40] at 230 cm^{-1} (**Figure S4**). No SERS effect for ferricyanide was detected at these potentials due to the presence of AgCl particles, which are neither active nor capable of producing the enhancement by the surface plasmon [41,42].

[FIGURE 2]

Although some SERS effect has been reported for pyridine on Ag^+/AgCl particles [43,44], there is no evidence of having such effect for ferricyanide in these experimental conditions, due to the Cl^- excess in solution, which precipitate immediately any Ag^+ cation. Increasing the electrode roughness is a factor considered as fundamental to produce a significant SERS effect [1,23]. However, these results demonstrate that the surface needs to have specific properties to produce the SERS effect (excitation of surface plasmons [45]), and, in this case, the roughness itself is not sufficient. From 0 V onwards, a wide and intense cathodic process, attributed to the reduction of the previously formed AgCl, was observed (**Figure 2A, blue line**). This cathodic process (between 0 V and -0.40 V) and the initial anodic process (between +0.30 V and 0 V) exchange a similar charge (2717 vs 2678 μC , respectively), suggesting that all the oxidized silver is subsequently reduced, generating a new silver roughened electrode surface. The Raman baseline increased drastically with a maximum intensity at a potential of -0.18 V (**Figure S3**), and quickly decreased afterwards (returned to the previous values at a potential of -0.25 V). An increment of the baseline intensity is usually attributed to fluorescence of some species, and therefore, in this case, it could be tentatively assigned to this process enhanced by the initial growing of Ag NPs (metal

enhanced fluorescence [46]) or the coexistence of fresh silver and a thick layer of AgCl. Simultaneous with the decreasing of the baseline, a Raman band at 2078 cm^{-1} began to grow from -0.19 V onwards (**Figure 2A, red line**) next to a lower-intensity band at 2030 cm^{-1} (observed as a shoulder) [47,48]. The use of TR experiments allows us to determine the exact time where a maximum SERS effect is detected, which in this case is reached between -0.30 and -0.40 V , just after the silver reduction process has finished. SEM image (**Figure 2D**) obtained at -0.35 V demonstrate that only Ag NPs are observed on the electrode surface (composition confirmed by EDX analysis, **Figure S5A**). Thus, NPs of different sizes with approximated spherical geometry were obtained in these conditions with an average diameter of $176 \pm 100\text{ nm}$ (distribution histogram in **Figure S5B**) and with a particle density around $4.5 \times 10^8\text{ NPs/cm}^2$. The *in situ* generation of these fresh NPs led to the significant SERS effect as has been demonstrated in earlier works [29,49].

Video S2 displays the TR-SERS SEC measurements recorded during this experiment (from 160 to 2800 cm^{-1}), which clearly highlights the usefulness of the technique to get dynamic time-resolved information able to show slight changes in the SEC response that could be used to study complex mechanisms occurring on the surface of SPEs and correlate them with structural properties.

The SERS spectra could be tentatively assigned to ferricyanide, since, in normal Raman, only emerges one band at 2125 cm^{-1} and ferrocyanide spectrum shows two bands at 2049 and 2086 cm^{-1} (**Figure S2**). However, it is interesting to study whether the initial ferricyanide is converted to ferrocyanide in the working potential window with silver electrodes. SEC experiments were performed with higher ferricyanide concentrations and by starting the voltammetry at a potential where the silver is not oxidized (to avoid overlapping in the voltammetric response due to the silver electrochemical processes with high currents). Voltammograms obtained at rising ferricyanide concentrations show an increasing cathodic process with a peak potential between -0.19 and -0.28 V (**Figures 3A**). Additionally, the evolution of the Raman spectra for 0.025 M (**Figure 3B**) suggests that the reduction of ferricyanide to ferrocyanide

takes place at a potential close to the reduction of silver, so the Raman signals obtained are mainly due to ferrocyanide, or probably, to some kind of prussian blue derivate generated on the electrode surface during the electrochemical reduction [50–52]. A band around 500 cm^{-1} is also observed during the TR-SERS SEC experiments (purple line, Figure S3), which is in agreement with similar bands found previously for prussian blue in presence of silver structures [53]. Therefore, it is reasonable to assume that prussian blue would be also formed on the electrode surface under these conditions. If this was the case, as prussian blue have a absorption band close to 700 nm [53] and the laser excitation is 785 nm , it could be possible the influence of the resonance Raman effect, and the spectra obtained in the SERS experiments would be due to surface-enhanced resonance Raman scattering (SERRS). **Video S3** shows the TR-Raman SEC experiment of the transformation of ferricyanide to ferrocyanide (with possible prussian blue generation). Initially, a band appeared at 2125 cm^{-1} (assigned to ferricyanide) with a small band at 2096 cm^{-1} (possibly due to some interaction with silver and loss of the molecule symmetry in comparison with ferricyanide in solution). Two new bands increased quickly at others Raman shifts (2030 cm^{-1} and 2083 cm^{-1}) closer to the values of the SERS signals. Furthermore, some interaction of the generated product with the silver surface is demonstrated in the previous SERS experiment since the signals are slightly shifted and the band at 2030 cm^{-1} is much smaller than that expected for ferrocyanide in solution.

[FIGURE 3]

Analytical performance of EC-SERS silver SPEs for ferricyanide detection

The analytical performance of the *in situ* activated silver SPEs was evaluated for the detection of low concentrations of ferricyanide. In this case, an integration time of 2 s was used to detect a greater number of photons than in the previous studies and, thus, to detect lower concentrations of the species.

However, 2 s is still a quite low integration time comparing to the typical quantitative Raman experiments. As was demonstrated before, the spectrum with the higher-intensity bands was that one registered at potentials near -0.35 V, when all the silver has been totally reduced. The intensity of the peak at 2078 cm^{-1} of the optimal spectrum was chosen as the analytical signal because is directly correlated to the initial ferricyanide concentration. **Figure 4A** shows the variation of the spectra with the concentration and **Figure 4B** shows the relationship between ferricyanide concentration and the peak intensity in a range between $5 \cdot 10^{-8} - 2.5 \cdot 10^{-5}$ M. At low ferricyanide concentrations, the peak intensity increases linearly with the concentration, but the response becomes saturated at higher concentrations. This behavior seems to follow a Freundlich-like adsorption isotherm as demonstrated by the good correlation observed in the **Figure 4B**. This adsorptive behavior can be explained because the SERS substrate has a defined number of active sites and the SERS response depends on the surface coverage of those sites as described by other authors [54,55]. The total saturation would be observed when all the active sites are occupied. This fact may also be in agreement with the adsorption of prussian blue on the electrode surface. The limit of detection calculated as the concentration given by three times signal-to-noise ratio was $1.5 \cdot 10^{-8}$ M. This limit of detection is well below the one that can be obtained using only electrochemical measurements [56,57]. The use of low-cost disposable SPEs with the dynamic electrochemical activation and *in situ* acquisition of Raman spectra allows the detection of very low concentrations of ferricyanide in a straightforward way and in only few seconds. The precision between measurements (using different electrodes) was excellent for a SERS-based assay, obtaining 8.4% as the RSD ($n=7$, for $2.5 \cdot 10^{-6}$ M). This demonstrates the reliable performance of the methodology employed in this work to make quantitative measurements by the TR-SERS SEC experiments, taking advantage of the SERS effect.

[FIGURE 4]

Analytical performance of EC-SERS silver SPEs for $[\text{Ru}(\text{bpy})_3]^{2+}$ detection

The *in situ* TR-SERS SEC method with the dynamic activation of the silver SPE is useful for the sensitive detection of different species. This is demonstrated by performing the detection of $[\text{Ru}(\text{bpy})_3]^{2+}$, which is usually employed as a spectroelectrochemical model or as a detection label in ECL assays [34]. The Raman spectra of $[\text{Ru}(\text{bpy})_3]^{2+}$ also varied dynamically with the potentials applied during the voltammetry performed for the surface activation as illustrated in the **Figure 5A**, which shows the variation of the intensity of the Raman band at 1032 cm^{-1} with the potential. This experiment was also carried out with a 0.250 s integration time and using $5 \cdot 10^{-7}\text{ M}$ of $[\text{Ru}(\text{bpy})_3]^{2+}$ in 0.1 M KCl and applying a linear-sweep voltammetry between +0.30 and -0.40 V at 0.05 V s^{-1} . However, in this case, there is no faradaic process along the potential sweep, because the electrochemical reaction of $[\text{Ru}(\text{bpy})_3]^{2+}$ take place at higher potentials. Therefore, the spectra obtained during the experiment are due to the initial species.

The TR-SERS SEC method was used to evaluate the quantitative detection of $[\text{Ru}(\text{bpy})_3]^{2+}$ by recording the band intensity at 1032 cm^{-1} of the most intense SERS spectrum at different concentrations of the analyte (**Figure 5B**). In this case, the response was only studied at low concentrations to avoid the saturation of the active sites as observed in the previous experiment. **Figure 5C** shows the calibration plot obtained with a linear range between $5 \cdot 10^{-8}$ and $2 \cdot 10^{-6}\text{ M}$ and the following linear equation:

$$I_p (\text{counts}) = 12.4 + 29246 \cdot \{[\text{Ru}(\text{bpy})_3]^{2+}\}(\mu\text{M}) \quad (\text{equation 3})$$

The detection limit calculated as the concentration given by three times signal-to-noise ratio was $2.1 \cdot 10^{-8}\text{ M}$. The precision of the assay, determined by measuring five times the solution of $2 \cdot 10^{-6}\text{ M}$ was 7.8% (RSD). These results demonstrate that the dynamic electrochemical activation of the silver SPEs with *in situ* detection of the SERS spectra is a powerful method for the sensitive, precise and rapid detection of different analytes. It is interesting to highlight the good precision obtained in these quantitative SERS assays, which could be tentatively ascribed to several factors: the Raman probe records the average optical response with a spot diameter of around $200\text{ }\mu\text{m}$, which reduces the influence of small heterogene-

ous features (hot spots or individual nanoparticles with different activity). Screen-printed electrodes are mass-fabricated using the reliable thick-film technology in a reproducible way, and their disposability avoids the need to manually clean and regenerate the silver surface as it happens for conventional electrodes. Also, the use of the robust spectroelectrochemical Raman cell with specific positions for the electrode and the probe ensures that the distance and angle remain constant between experiments.

[FIGURE 5]

CONCLUSIONS

In the present work, we have studied dynamically with a high time-resolution the *in situ* electrochemical activation of silver SPEs, in order to generate a SERS-active substrate for *in situ* quantitative purposes with a high sensitivity and reproducibility. We have recorded in real-time the Raman SEC data of the processes occurring at the silver SPEs using low integration times. The good correlation between the SEC data and the microscopic structural features provided relevant information about the activation process of Ag electrodes (widely employed for SERS-based assays), in order to deepen into the *in situ* SERS substrate generation. TR-SERS SEC has been exploited for the analytical determination of ferri-cyanide and $[\text{Ru}(\text{bpy})_3]^{2+}$ at very low concentrations (15-21 nM with an integration time of only 2 s), which demonstrates the outstanding usefulness of the novel SEC method with disposable low-cost electrodes for the detection of analytes in extremely low concentrations. Besides, the quality of the analytical results (LOD, LOL and RSD) are fairly good compared with those usually obtained for normal quantitative Raman experiments, which enhances the importance of this technique in analytical quantitative applications. Finally, from this work, it will be possible to design TR-SEC methods to quantify a

large number of analytes, taking advantage of the great potential of the Raman technique, combined with the high sensitivity enhanced by the SERS effect.

ACKNOWLEDGMENTS

Financial support from Ministerio de Economía y Competitividad (CTQ2014-61914-EXP, CTQ2014-55583-R, TEC2014-51940-C2-2R, CTQ2015-71955-REDT) and Junta de Castilla y León (BU033-U16) is gratefully acknowledged. J.V.P. thanks Junta Castilla y León for his postdoctoral fellowship (BU033-U16).

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FIGURES

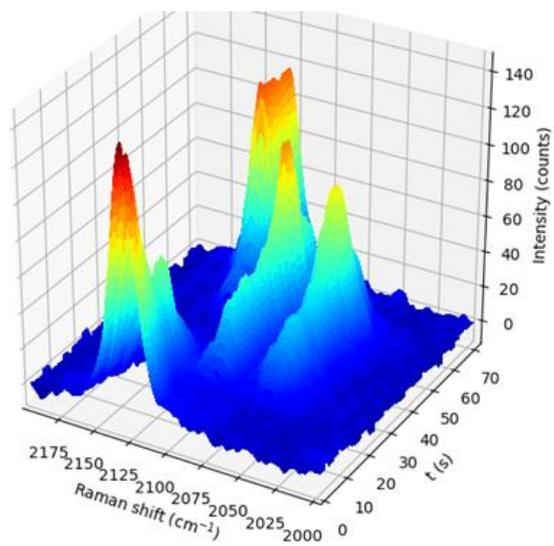


Figure 1. Surface plot of the Raman spectra evolution with the time (related to the applied potential) obtained for the spectroelectrochemical experiment using an initial concentration of 0.05 M of ferricyanide in 0.1 M KCl and screen-printed carbon electrodes.

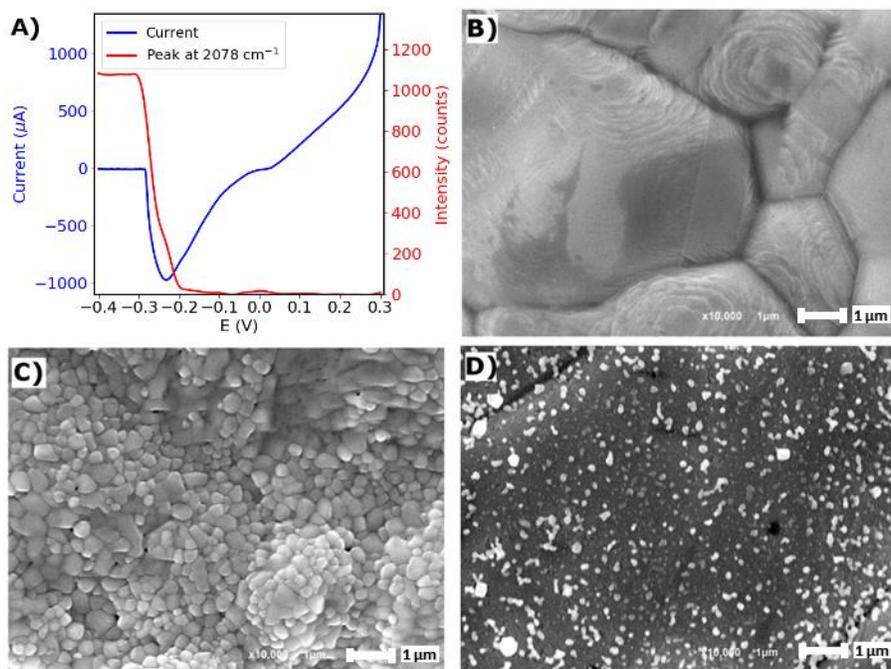


Figure 2. A) Evolution of the Raman peak intensity (baseline corrected) at 2078 cm^{-1} (red line) during the linear sweep voltammogram (blue line) performed to activate the surface of screen-printed electrodes. A solution of 10^{-5} M of Ferricyanide in 0.1 M KCl was used. Integration time was 0.250 s . SEM image of the surface of screen-printed silver electrodes at **B)** the initial stage (pristine electrode), **C)** after performing the electrochemical pretreatment from $+0.30$ to $+0.05\text{ V}$ and **D)** after performing the *in situ* electrochemical activation stopping the potential at -0.35 V . Scale bars are $1\text{ }\mu\text{m}$ in all images.

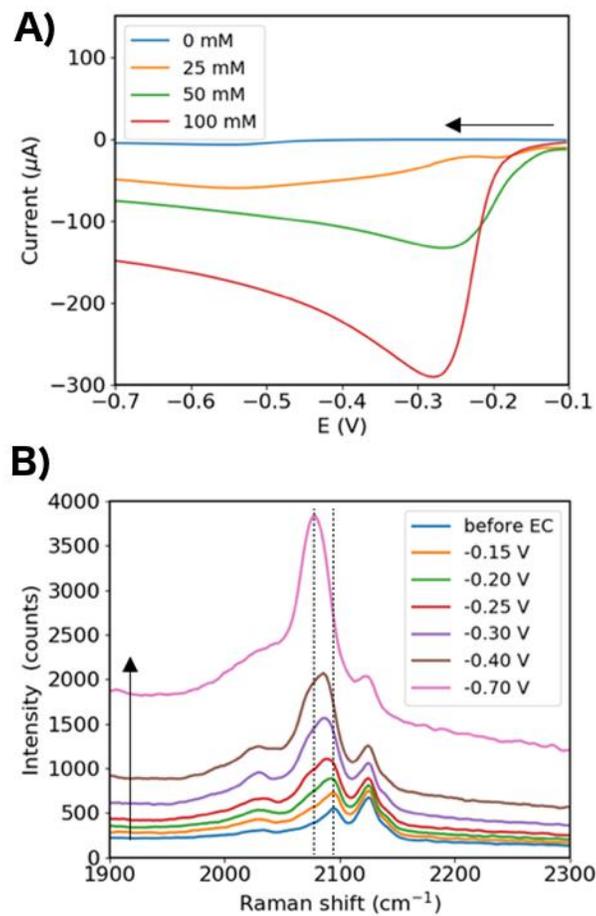


Figure 3. **A)** Linear sweep voltammetry, starting from -0.10 V towards negative potentials, obtained for solutions containing 0, 0.025, 0.05 and 0.1 M of ferricyanide in 0.1 M KCl, using silver screen-printed electrodes. **B)** Potential-dependent evolution of the Raman spectra for a solution of 0.025 M ferricyanide in 0.1 M KCl.

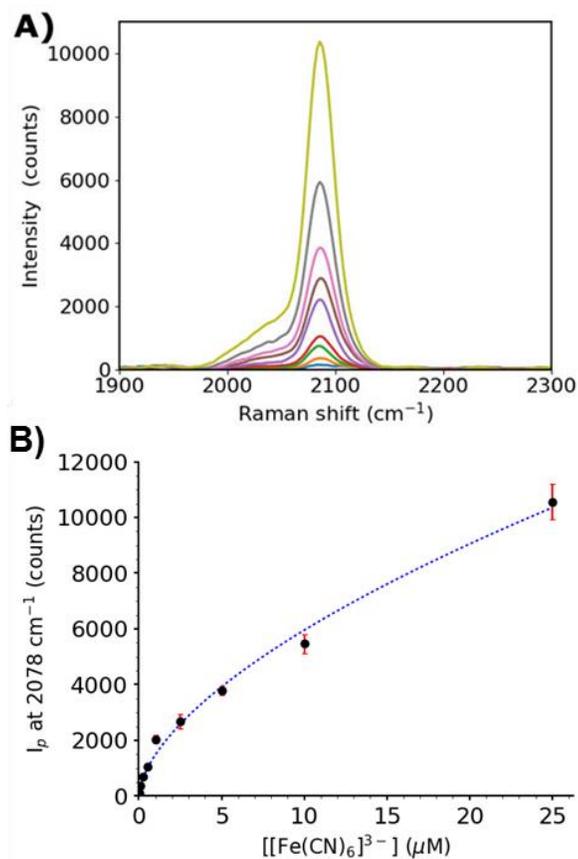


Figure 4. **A)** EC-SERS spectra (baseline corrected) obtained at the optimum potentials (between -0.3 and -0.4 V, integration time: 2 s) for different ferricyanide concentrations (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 25 μM). **B)** Relationship between the Raman peak intensity at 2078 cm⁻¹ for the EC-SERS spectra and the concentration of ferricyanide in 0.1 M KCl. Dashed blue line shows the fitting of a Freundlich adsorption isotherm.

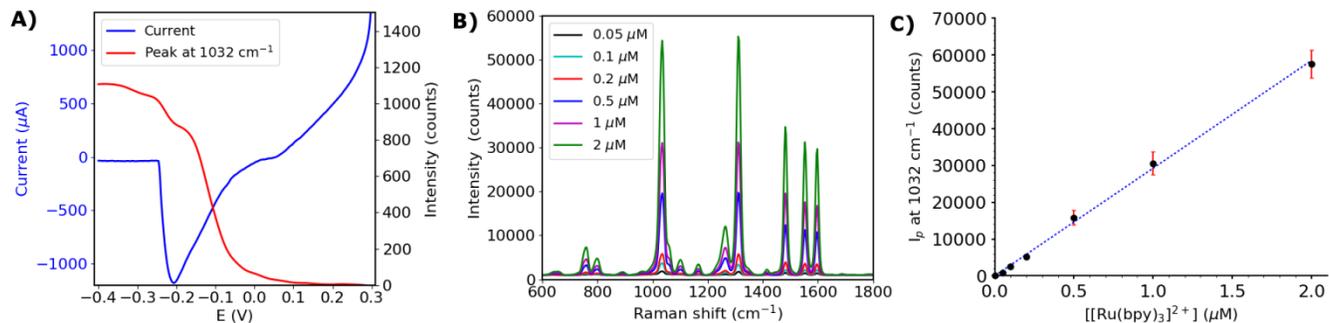


Figure 5. **A)** Evolution of the Raman peak intensity at 1032 cm⁻¹ (red line) during the linear sweep voltammogram (blue line) performed to activate the surface of screen-printed electrodes. A solution of 0.5 μM of [Ru(bpy)₃]²⁺ in 0.1 M KCl was used. Integration time was 0.250 s. **B)** EC-SERS spectra (baseline corrected) obtained at the optimum potentials (between -0.3 and -0.4 V, integration time: 2 s) for different [Ru(bpy)₃]²⁺ concentrations (0.05, 0.1, 0.2, 0.5, 1 and 2 μM). **C)** Linear relationship between the Raman peak intensity at 1032 cm⁻¹ for the EC-SERS spectra and the concentration of [Ru(bpy)₃]²⁺ in 0.1 M KCl.

SUPPORTING INFORMATION

Quantitative Raman Spectroelectrochemistry using silver screen-printed electrodes

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FIGURES

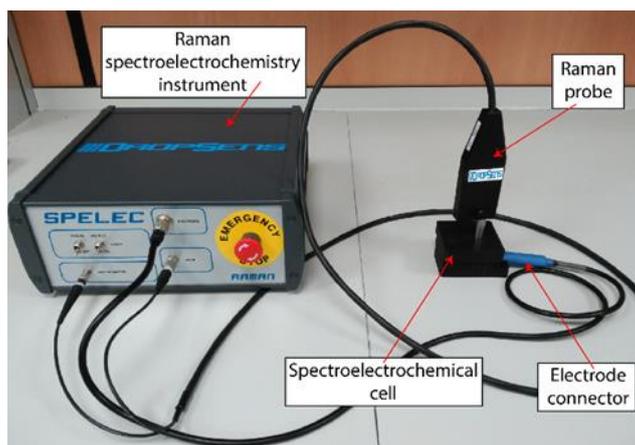


Figure S1. Picture of the instrumentation employed for Raman spectroelectrochemistry with screen-printed electrodes.

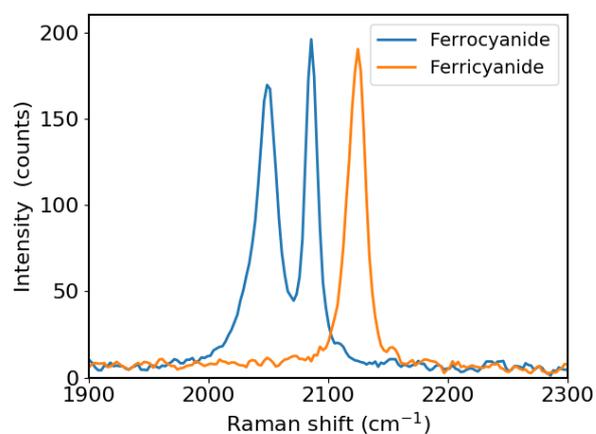


Figure S2. Raman spectra obtained for solutions of 0.1 M of ferro- and ferricyanide in 0.1 M KCl. Raman spectra for the solid products were qualitatively similar.

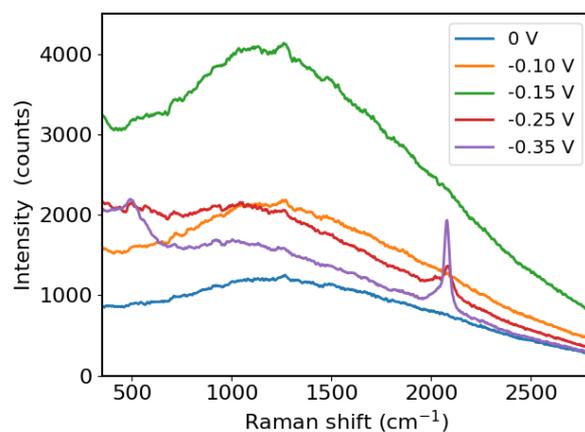


Figure S3. Raman spectra of 10^{-5} M ferricyanide in 0.1 M KCl at different potentials during the linear sweep voltammetry from +0.30 V to -0.40 V at 0.05 V s^{-1} in a silver screen-printed electrode.

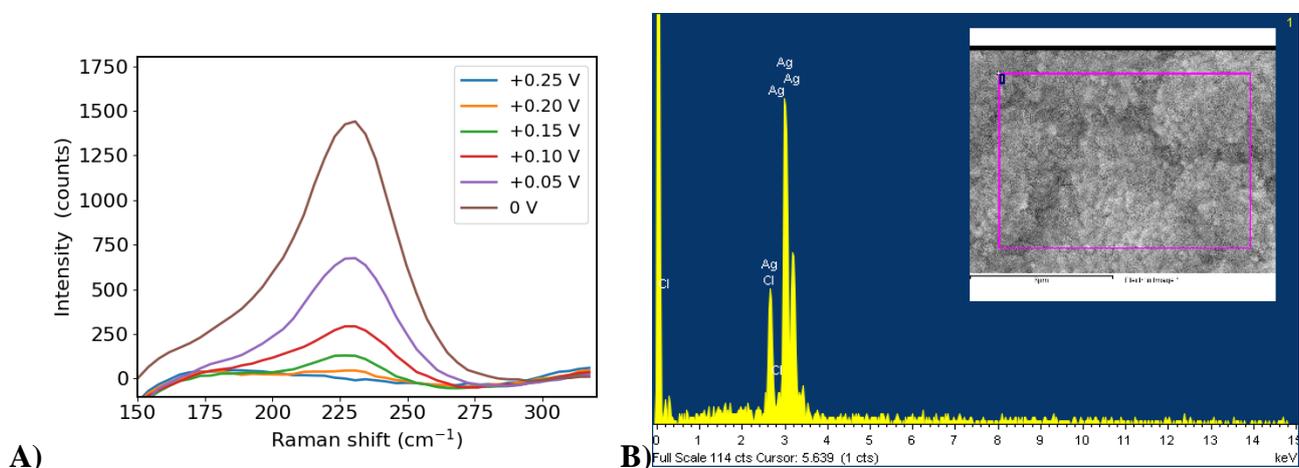


Figure S4. A) Evolution of the Raman spectra from 150 to 300 cm^{-1} at different potentials during the *in situ* electrochemical activation. **B)** EDX spectrum of the silver electrode surface after performing the electrochemical pretreatment of a silver screen-printed electrode from +0.30 to +0.05 V in presence of 10^{-5} M ferricyanide in 0.1 M KCl.

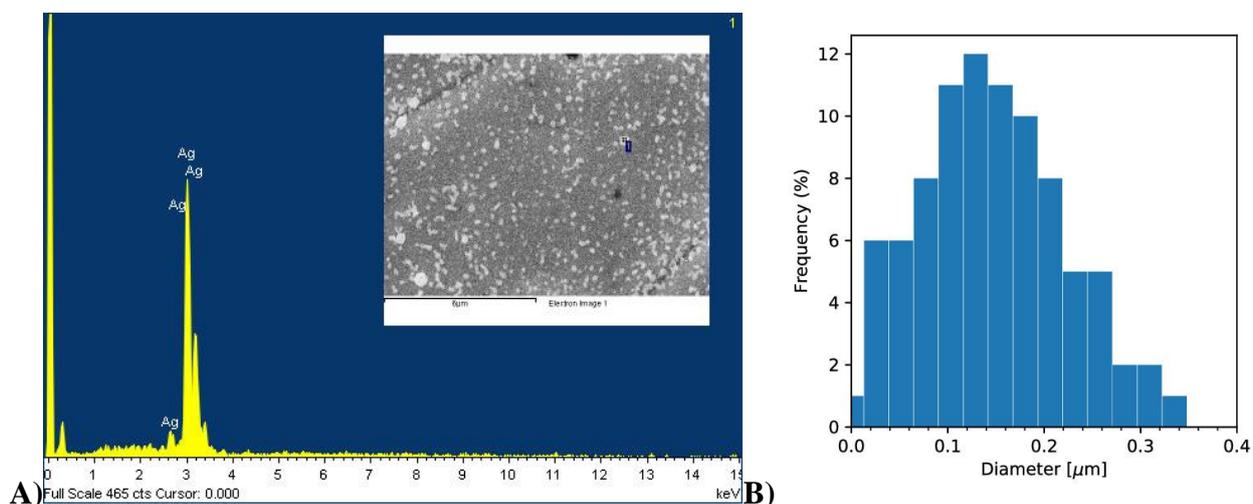


Figure S5. **A)** EDX of a nanoparticle found on the electrode surface after the electrochemical activation by linear sweep voltammetry (stopping at -0.35 V). Only Ag bands are found in the spectrum, which indicates that chloride has been removed from the surface. Inset shows the location of the nanoparticle in the SEM image. **B)** Histogram illustrating the size distribution of the silver nanoparticles found on the SEM images stopping the electrochemical activation of a silver screen-printed electrode at -0.35 V.

VIDEOS

Video S1. Dynamic evolution of the Raman spectra for 0.05 M ferricyanide in 0.1 M KCl during the cyclic voltammetry experiment using screen-printed carbon electrodes.

Video S2. Dynamic evolution of the Raman spectra for 10^{-5} M ferricyanide in 0.1 M KCl during the cyclic voltammetry experiment using screen-printed silver electrodes. Inset shows the cyanide region where the SERS signal for ferri/ferrocyanide species is observed.

Video S3. Dynamic evolution of the Raman spectra for 0.025 M ferricyanide in 0.1 M KCl during a cyclic voltammetry experiment starting at -0.10 V using screen-printed silver electrodes.