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Highly reproducible SERS detection in sequential injection analysis: Real time preparation and application of photo-reduced silver substrate in a moving flow-cell

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ABSTRACT

This paper reports an improved way for performing highly reproducible surface enhanced Raman scattering of different analytes using an automated flow system. The method uses a confocal Raman microscope to prepare SERS active silver spots on the window of a flow cell by photo-reduction of silver nitrate in the presence of citrate. Placement of the flow cell on an automated x and y stages of the Raman microscope allows to prepare a fresh spot for every new measurement. This procedure thus efficiently avoids any carry over effects which might result from adsorption of the analyte on the SERS active material and enables highly reproducible SERS measurements. For reproducible liquid handling the used sequential injection analysis system as well as the Raman microscope was operated by the flexible LabVIEW based software ATLAS developed in our group. Quantitative aspects were investigated using $Cu(PAR)_2$ as a model analyte. Concentration down to 5×10^{-6} M provided clear SERS spectra, a linear concentration dependence of the SERS intensities at 1333 cm^{-1} was obtained from 5×10^{-5} to 1×10^{-3} with a correlation coefficient $r=0.999$. The coefficient of variation of the method V_{xo} was found to be 5.6% and the calculated limit of detection 1.7×10^{-5} M. The results demonstrate the potential of SERS spectroscopy to be used as a molecular specific detector in aqueous flow systems.

1. Introduction

Surface enhanced Raman scattering (SERS) has attracted a lot of attention in recent years because it allows highly sensitive and molecular specific measurements of different types of analytes. Successful application can be found in many different areas ranging from life sciences to environmental monitoring as well as biomedical diagnostics to name a few [1–3]. SERS is observed when the analyte of interest is in close vicinity or adsorbed on rough surfaces of noble metals such as silver or gold. As the observed enhancement factors strongly depend on the geometry and the structure of the SERS material, a lot of research efforts have been devoted to this subject in recent years [4–8]. Important goals of the performed activities are optimization of the SERS active structures to maximize the enhancement factors as well as the integration of SERS in biomedical assays for maximum selectivity also in case of complex samples. Another aspect to consider, especially when discussing the applicability of SERS from a practical point of view is the robustness of the detection method itself. In SERS this not only includes the shelf life of prepared SERS substrates such as colloids or especially designed nanostructured particles or arrays. Of equal importance is the way SERS detection is implemented in a given analytical protocol. Among the different possibilities to do so, the combination of SERS and automated flow analysis, either in a standard or miniaturized format is a popular option [9–11]. One reason for this is the need for a destruction free, but highly sensitive molecular specific detector in separation techniques [12] as well as lab-on-a-chip type microfluidic systems [13], where SERS is being regarded as a potential candidate for highly sensitive detection [14–18]. The miniaturization of reaction systems offers practical advantages over the conventional bench-top systems. Methods have been proposed to enhance the signal from such experimental systems and to improve detection limits. Such techniques have included the use of mechanical traps to aggregate the colloid at a narrowing of the channel, in order to concentrate the analyte as it flows over the colloidal particles [19,20] and, hence, improving the SERS signal.

Automated flow systems are a very versatile platform for carrying out sequences of highly reproducible sample and reagent

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manipulations. These sequences may include many different unit operations including reproducible manipulation of beads [9,21] as well as levitated droplets [22]. In previous work, we showed that in the case of preparation and application of hydroxylamine reduced silver sols, important benefits in terms of precision and robustness of the overall measurement protocol could be achieved. This is mainly due to the fact that for each subsequence measurement a new SERS active surface was prepared. A common characteristic of popular methods which are based on a chemical reduction of silver nitrate to produce SERS active silver surfaces, either using citric acid [23], boron hydride [10,24,25] or hydroxylamine [26], is the release of gaseous reaction products which are produced during the reduction step. The release of these gases makes it difficult to implement these chemical SERS preparation techniques in the closed conduits of flowing systems. A way to overcome these issues related to gas release during silver reduction is to prepare SERS active silver spots by photo reduction. This technique is based on a work by Bjerneld et al. [27] who showed that the photochemical reduction of silver nitrate is possible. Upon illumination with light a mixture of silver nitrate and citric acid reacts to colloidal silver as well as acetone-1,3-dicarboxylate as well as carbon dioxide [28,29]. Ag photo-reduction has been used previously to generate silver nanoparticles (AgNPs) by means of laser photo-reduction of a silver nitrate solution [30,31]. This approach was then also used for SERS detection in capillary electrophoresis [32]. In this application the HeNe laser of the confocal Raman spectrometer was used to produce the required SERS colloid as well as to record SERS spectra of the analytes. Due to the fact that only a very small amount of silver is reduced the amount of released carbon dioxide is small and thus readily dissolved in the flowing liquid. Therefore, preparation of SERS substrates by photo-reduction is well compatible with in-line SERS detection in flow systems. This was shown later in a follow-up paper by Herman et al. [33] where repeated analysis of different samples in a flow injection system was successfully demonstrated. Common to both papers was the addition of silver nitrate and citrate to the buffer system in case of electrophoresis and to the carrier solution in case of flow injection system. However, as can be seen from both papers, memory effect remained a serious problem. This was because SERS substrate preparation and application was always carried out at the same spot.

This paper describes a, what we believe, significant improvement as it solves the problem of memory effects in flow systems efficiently. First, we propose to turn to sequential injection analysis for improved computer of the whole system. Second, and more relevant, we propose to take advantage of the small spot size of the photo-reduced SERS active silver spot and to move the flow cell where SERS detection is to be performed a few micrometre after each measurement. Third, for fast preparation of a SERS active spot, we redesigned the flow cell and suggest the use of a flow cell made up by two planar CaF_2 windows. In this case a faster SERS spot synthesis can be achieved and continued alignment of the detection unit, despite movement of the flow cell, can be realized.

2. Expermintal

2.1. Experimental setup and used instumentation

A schematic view of the experimental setup is shown in Fig. 1. The sequential injection analysis system (SIA) consisted of a 5 ml syringe pump of Cavro Scientific Instruments (Sunnyvale, CA, USA), a 6-port selection valve of VICI (Valco Instruments Co. Inc.) and of PTFE tubings with an internal diameter (i.d.) of 0.75 mm. For Raman measurement the SIA system was connected to a flow-

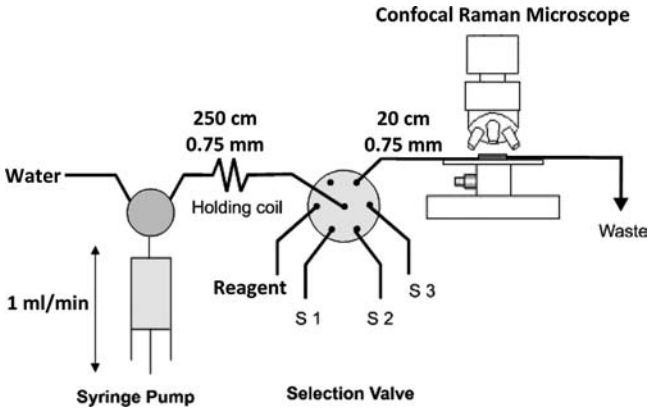

Fig. 1. Schematic view of the experimental setup for automated multianalyte sequential SERS detection in a flow cell.

cell, initially designed for mid-IR spectroscopy, which consisted of two 2 mm thick rectangular Calcium Flouride (CaF_2) windows which were separated by a 200 μm thick PTFE spacer.

The flow cell was placed on the computer controlled x, y, and z, stages EK32 75 × 50-0.4 mm (Märzhäuser Wetzlar GmbH, Germany) of the confocal Raman spectrometer LabRam HR800(Horiba Jobin Yvon, Bensheim, Germany) which is based on an Olympus B × 41 optical microscope.

For recording SERS spectra a HeNe laser line (632.8 nm), a Nikon objective (× 20, NA 0.35, and WD 20.5) and a 600 lines/mm grating were used along with a charge coupled device detector (CCD). Furthermore, neutral density filters were used to attenuate the HeNe laser beam. The slit width was set to 100 μm and spectra in the relevant range from 540 to 1660 cm^{-1} were recorded using a 2 s integration time.

For full computer control of the whole experimental set-up, including SERS spectrum acquistion, we used our in-house developed LabVIEW based ATLAS software [34] to which, as a new feature, remote operation of the LabRAM sepectrometer was added.

Characterization of the prepared SERS substrate was done with scanning electron microscopy (SEM). For this purpose SEM images were obtained with a FEI Quanta 200 (FEI Company, Eindhoven, The Netherlands). Images were performed with electron beam energy of 15 keV and detecting back scattered electrons.

2.2. Chemicals

4-(2-Pyridylazo)resorcinol (PAR), rhodamine 6G (R6G), methylene blue (MB), copper(II) sulphate pentahydrate, sodium citrate dihydrate and silver nitrate were of analytical grade. Deionized water was used to prepare the solution and as a carrier liquid. PAR complexes with Cu(II) were prepared by mixing solutions of 10^{-3} M copper(II) sulphate pentahydrate and PAR at 1:1 M ratios, resulting in $Cu(PAR)_2$ complexes, PAR forming bidentate complexes with Cu(II) [35].

For preparation of the SERS active silver spot an aqueous solution containing 0.5 mM silver nitrate and 5 mM sodium citrate with a pH of 7.5 was used. This solution was prepared freshely each day.

3. Results and discussion

The aim of this study was the development of a fast measurement protocol for highly precise SERS measurements of different analytes void of memory effects, using the proposed combination of SIA with automated preparation and use of photo-reduced silver spots for SERS detection.

As both preceeding papers [32,33] reported the use of the Cu $(PAR)_2$ complex as a model analyte and because of its strong memory effects, this analyte was used for testing of the whole system. The memory effects of the $Cu(PAR)_2$ resulted in significant peak tailings in capillary elcetrophoresis and required extended rinsing (10 min) in flow injection analysis prior to a subsequent analysis.

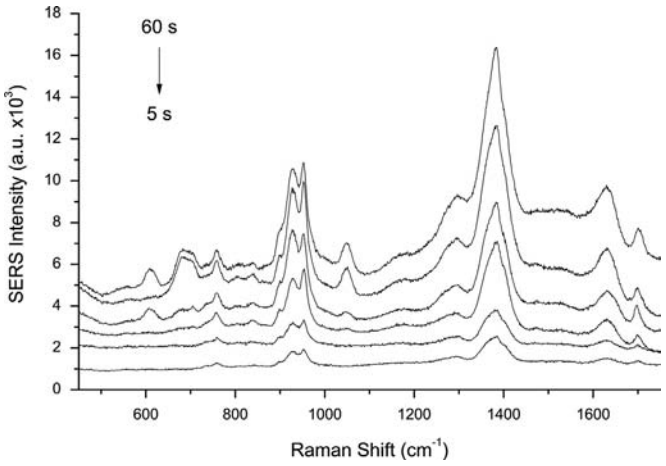

Fig. 2. SERS spectra of citrate anions on silver spot using different laser illumination times (5, 10, 20, 30, 40 and 60 s). The concentration of the reagent solution (0.5 mM silver nitrate and 5 mM sodium citrate).

During the experiments carried out in this study the flow cell was kept at a constant position for each sample injection and moved 50 μm upstream prior to the analysis of the next sample. First, 500 μl of the reagent solution required for preparation of the SERS active spots, which contained 0.5 mM $AgNO_3$ and 5 mM sodium citrate at pH 7.5, was introduced to the holding coil via the selection valve and pumped to the flow cell at a flow-rate 1 ml/min. During passage of the reagent solution the laser was turned on and focused on the surface of the lower CaF_2 window using the 20× magnification objective. During this time period, Raman spectra with an accumulation time of 2 s were recorded. Using these conditions, the formation of photoreduced silver spots could be observed already after an illumination time of 5 s. The formation of the SERS active spots also lead to SERS spectra of the citrate ions which were attached to the silver surface. The intensity of these spectra increased during SERS spot fabrication as can be seen from Fig. 2. In an effort to characterize these SERS active silver spots, a CaF_2 window carrying a number of spots produced by different laser illumination times were characterized by secondary electron microscopy (Fig. 3). The corresponding images confirmed the formation of rough silver surfaces as required for SERS spectroscopy.

After injection of the reagent solution the samples containing different analytes were injected again via the selection valve. As an example the sequential injection of the $Cu(PAR)_2$ complex (10^{-4} M), rhodamine 6G (10^{-5} M), and methylene blue (3×10^{-6} M) is shown in Fig. 4A. Due to the intensity in the recorded SERS spectra of these analytes the presence of citrate cannot be detected. For each analyte increasing SERS intensities are obseved as soon as the analyte enters the flow cell. Movement of the stage after 30 s enables measurement

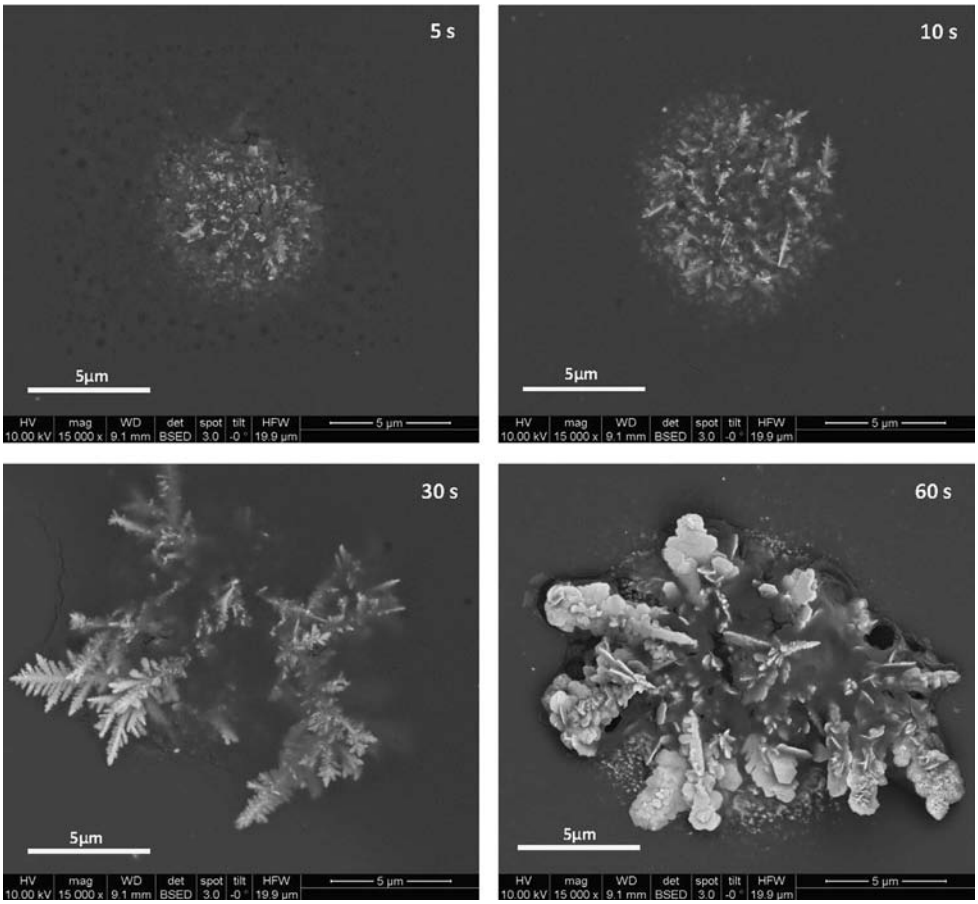

Fig. 3. SEM images of laser-induced silver substrates prepared on a CaF_2 window after 5, 10, 30 and 60 s of laser exposure.

of the subsequent injected sample independently of the one previously measured. From the obtained recording selected spectra have been extracted and depicted in Fig. 4B, to show the high quality of the obtained data, which clearly shows that it is indeed possible to avoid cross contamination and to record clear SERS spectra of three different analytes in less than 120 s.

The $Cu(PAR)_2$ complex was used in previous literature as a model analyte for flow injection analysis [33] and capillray electrophoresis [32]. In both applications this analyte exhibited a strong memory effect. In capillary elcetrophoresis this resulted in significant peak tailings. In case of flow injection analysis it required rinsing for up to 10 min to regenerate the SERS substrate for subsequent analysis. Therefore, this analyte was selected for detailed testing of the whole analysis system developed in this work.

During these tests the influence of the flow rate (Fig. 5A) and the sample volume (Fig. 5B) on the recorded intensities of the SERS spectra of $Cu(PAR)_2$ were investigated in the range of 0.5–3.5 ml/min and 5–60 μl, respectively. As may be seen in Fig. 5A, the slower the flow rate, the more intense is the SERS spectrum of the analyte. This clearly shows that dynamic processes are involved that govern the overall observed SERS intensities. One such process is the establishment of the adsorption equilibrium of the analyte on the silver substrate [10]. A second contribution to this behaviour is seen in the fact that strongly laminar flow condition prevail in the flow-cell, thus the only process which brings the analyte molecules close to the SERS surface is diffusion. Therefore, an increased residence time in the flow cell which is achieved at lower flow-rates will allow more analyte molecules to get close to the SERS active silver spot.

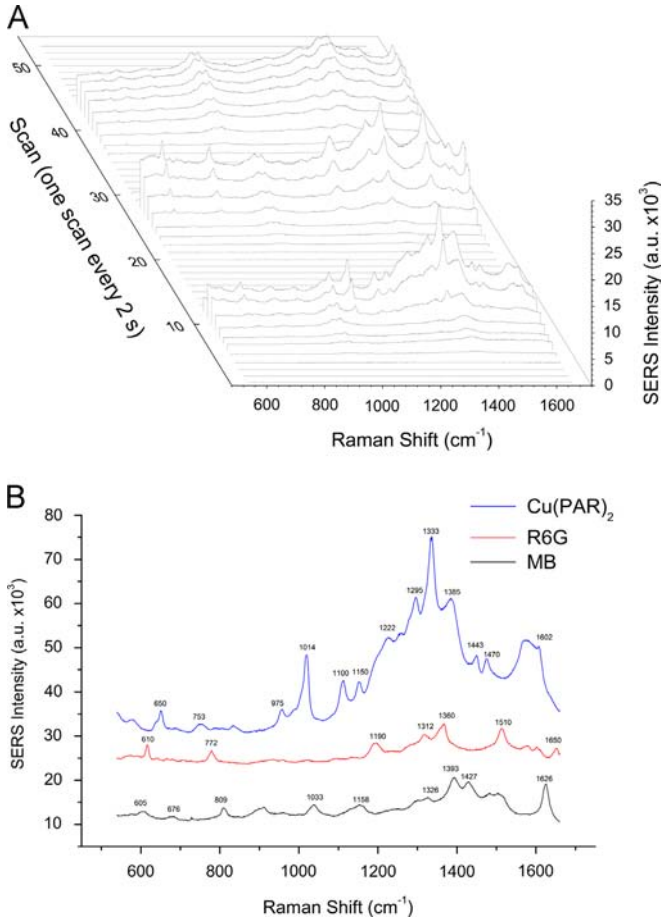

Fig. 4. (A) Sequentially recorded SERS spectra of the 10^{-4} M $Cu(PAR)_2$, 10^{-5} M R6G and 3×10^{-6} M MB. The flow rate was 1 ml/min. (B) SERS spectra of the three analytes in the flow cell.

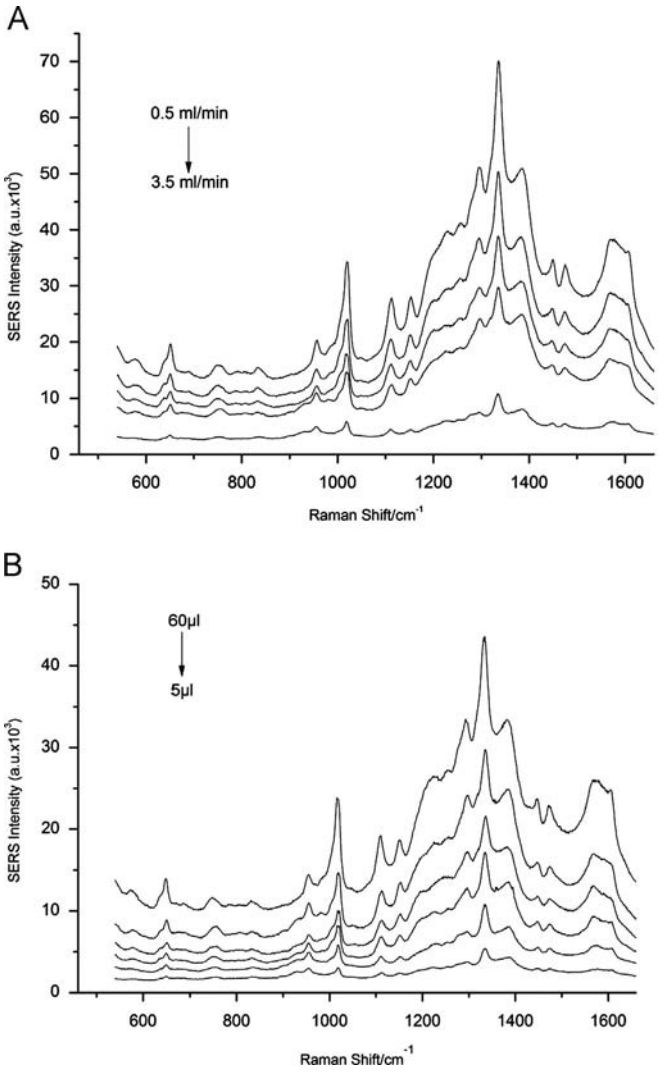

Fig. 5. (A) SERS spectra of 30 μl (10^{-4} M) $Cu(PAR)_2$ complex using 3.5, 2.8, 2.5, 1 and 0.5 ml/min flow rates. (B) SERS spectra of different volumes of the 10^{-4} M $Cu(PAR)_2$ complex using1 ml/min flow rate. The volumes are 5, 10, 20, 30, 40 and 60 μl.

Table 1
SERS intensities at 1333 cm^{-1} of the $Cu(PAR)_2$ complex during repeated analysis.

No.	SERS Intensity[a] (arbitrary units) × 10^3					
	5×10^{-6} M	1×10^{-5} M	5×10^{-5} M	1×10^{-4} M	5×10^{-4} M	1×10^{-3} M
1	5.80	6.49	11.3	14.1	37.5	68.5
2	5.90	6.33	11.2	13.6	38.2	70.9
3	5.90	6.43	11.2	13.9	38.6	68.7
4		6.58		14.2	37.6	68.1
5		6.65		13.8	37.2	70.2
6		6.54		13.7	37.9	69.3
7		6.39		14.0	38.3	69.4
8		6.47		13.9	38.7	68.5
Mean	5.83	6.48	11.3	13.9	38.0	69.2
SD	0.0577	0.104	0.0577	0.200	0.539	0.954
RSD (%)	0.989	1.60	0.511	1.44	1.42	1.38

[a] Injected volume: 30 μl and flow rate: 1 ml/min.

The major point of this paper, however, concerns repeatabilty which can be achieved by this new approach. For this purpose six different concentrations of $Cu(PAR)_2$ were prepared and analysed. Four concentrations were injected eight times, whereas two concentrations were analysed in triplicate. In each case 30 μl were injected and a flow rate of a 1 ml/min used. For quantitative evaluation of the recorded SERS spectra the intensity of the strong band at 1333 cm^{-1} corresponding to in-plane bending of the benzene ring was selected [35]. The intensity readings at 1333 cm^{-1} for all injections are stated in Table 1. As can be seen from the calculated r.s.d. values a very high repeatability in the range of 0.51–1.6% was obtained throughout the studied concentration range. Statistic evaluation of the whole data set using the software programme Validata in Excel revealed that for a confidence level of 95% the linear range of the calibration curve extended from 3×10^{-5} to 1×10^{-3} mol/l [36]. The obtained calibration curve is shown in Fig. 6 together with representative SERS spectra of the individual $Cu(PAR)_2$ concentrations. Fig. 7 shows the full recording of eight repetitive injections of a the $Cu(PAR)_2$ complex at a concentration of 10^{-4} mol/l. It can be observed that highly repeatable recordings were obtained and that any memory effect could be avoided by having a new SERS active spot available for every subsequent analysis. The inset in Fig. 7 shows a picture of the created SERS spots which are equally distributed on the planar CaF_2 surface of the window of the flow cell at a distance of 50 μm. Due to the small spacing of the individual SERS spots and because of the width of the free space in the spacer of 10×20 mm a large number of individual measurements can be performed using one single CaF_2 window.

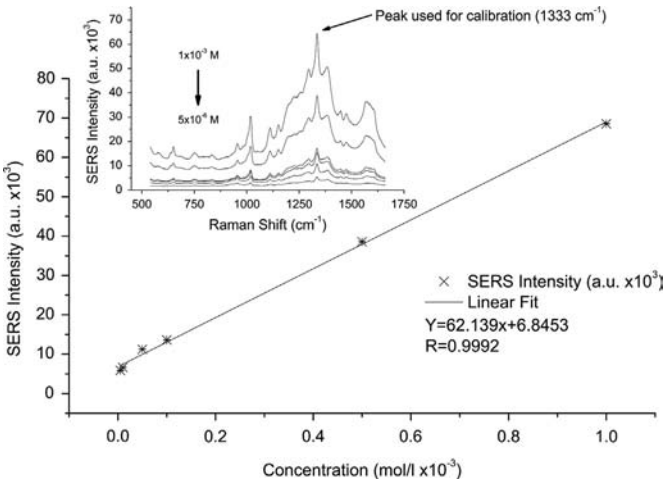

Fig. 6. Calibration curve of 30 μl $Cu(PAR)_2$ complex using 1 ml/min flow rate at 1333 cm^{-1} (the upper inset) SERS spectra of different concentrations 30 μl $Cu(PAR)_2$ complex (1×10^{-3}, 5×10^{-4}, 1×10^{-4}, 5×10^{-5}, 1×10^{-5} and 5×10^{-6} M). The flow rate was 1 ml/min.

4. Conclusion

This paper introduced a new way for highly repeatable SERS detection in automated flow systems such as in sequential injection analysis. We consider this approach an important step for establishing SERS as a reliable detection method in flow systems because it significantly improves the current state of the art in terms of robustness. The advantages of this simple procedure for SERS detection in flow systems are mainly due to full computer control of the flow system including the Raman spectrometer and its x, y, and z stages. This full computer control allowed the synthesis of new and thus clean SERS active silver substrates prior to each sample injection, a prerequiste for highly repeatable and fast analysis. In future work it is planned to expand on this technique and to evaluate its application also in separation systems such as liquid chromatography and electrophoresis.

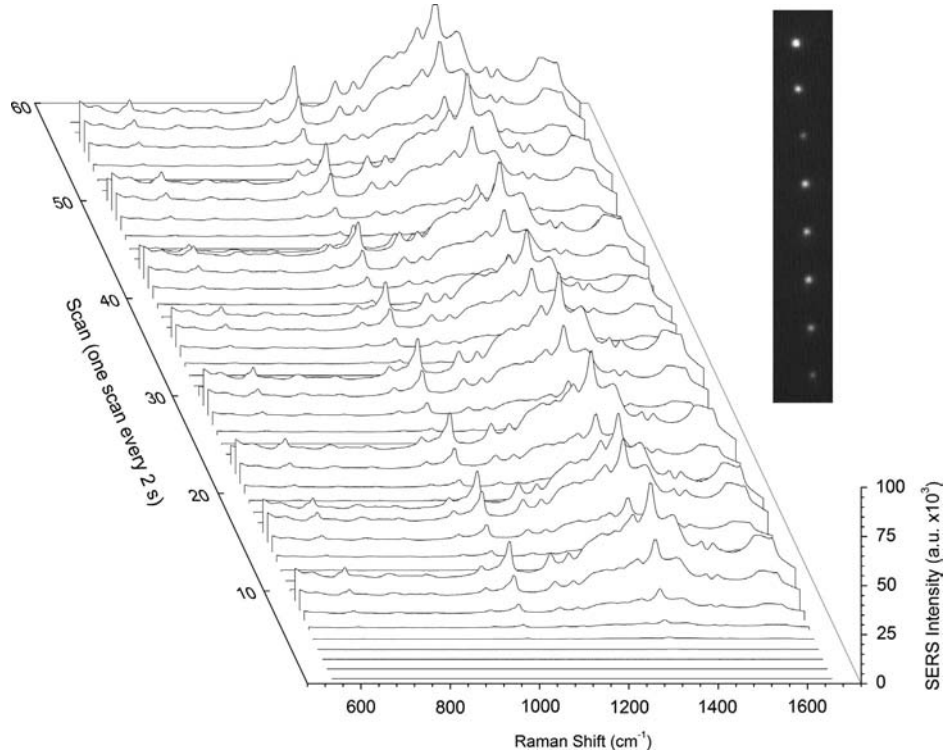

Fig. 7. Sequentially recorded SERS spectra of eight replicates of 30 μl (10^{-4} M) $Cu(PAR)_2$ complex using 1 ml/min flow rate, (the upper inset) Optical image of the laser-induced silver substrates in eight replicates for four concentration levels of the complex formed in the lower window of the flow cell.

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