SURFACE-ENHANCED RAMAN SPECTROMETRY FOR DETECTION IN LIQUID CHROMATOGRAPHY USING A WINDOWLESS FLOW CELL

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Summary—A windowless flow cell has been developed for surface-enhanced Raman (SER) detection in liquid chromatography. Using colloidal silver as an active substrate, SER spectrometry of banned drugs in sport is presented. The experimental factors of primary influence on the analytical performance of the cell have been studied. The results of SER detection with the proposed cell have been compared to UV detection.

Surface-enhanced Raman spectrometry (SERS) on colloidal systems is a sensitive, selective and fairly simple method for the analysis of a variety of compounds of technical, environmental, biomedical and pharmaceutical interest. The requirement for analyte-induced aggregation of the colloidal system introduces a range of experimental variables that are difficult to control. Driven by the excellent separative and sample handling performance of modern analytical flowing systems, colloid SERS systems have been adapted for detection in flowing streams (flow injection analysis (FIA) and high performance liquid chromatography (HPLC)).

In the earliest configuration,1,2 a relative standard deviation of 3% was reported for replicate injection of p-aminobenzoic acid on a stream of colloidal silver prepared on-line at room temperature. After these reports, other applications of colloid SERS on flowing streams were described.3-7 The separation and determination of four purine bases (adenine, guanine, hypoxanthine and xanthine) by reverse-phase HPLC in combination with real-time SERS have been demonstrated.8 Limits of detection in the nanomolar range were reported. In spite of these advantages, SERS detectors using conventional, closed flow cells have several drawbacks. Tailing in the chromatographic peaks results from memory effects caused by deposition of Ag sol and adsorbed compounds on the inner wall of the cell.9 To eliminate the memory effect, it is often necessary to change the flow cell or to rinse it with nitric acid after each chromatographic run. Adsorption on the cell walls also results in baseline drifts, contamination problems and mixing of the spectra of the previously separated compounds.

The need for a reliable but simple analytical device for detection in HPLC remains an important goal in SERS. Open flow cells have been proposed for detection in HPLC under a variety of analytical principles. A windowless cell was first proposed for laser-induced fluorescence detection.9 Using the 325-nm modulated output of a He-Cd laser and phase-sensitive detection, carcinogenic aflatoxins were linearly quantitated to $7.5 \times 10^{-13}$ g. Later, the fluorescence and photoacoustic characteristics of an open flow cell intended for liquid chromatographic applications were compared with the respective characteristics of a static cuvette cell.10 The advantage of such a detector is that photoacoustic detection is complementary to fluorescence detection and may be useful in quantititating substances which do not appreciably luminesce. A laser two-photon ionization technique was applied for the trace determination of pyrene using a windowless cell made of two stainless steel electrodes.11 With 300-µl dead volume, the cell, in combination with a pulsed nitrogen laser, allowed the detection of 100 ng/ml pyrene in n-heptane. An open cell was developed that had simultaneous detection capabilities by molecular fluorescence, photoacoustic effect and two-photon photoionization processes. The cell was evaluated for drugs,12 aromatic compounds13 and polycyclic aromatic hydrocarbons.14 In the present paper we discuss the utility of SERS detection in HPLC using an

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open flow cell. The operating performance of the cell is illustrated for banned drugs in sport. The results of SER detection with the proposed cell have been compared to UV detection.

**EXPERIMENTAL**

**Instrumentation**

A schematic diagram of a coupled HPLC–SERS system is shown in Fig. 1. Spectra were obtained with a 0.22-m double-grating spectrometer (Spex Industries, model 1680B) with a 1200 grooves/mm classically ruled grating. The Raman scattering was collected at right-angles and detected with a cooled photomultiplier tube (Hamamatsu, model R-928), a fast preamplifier (Stanford Research Systems, model SR440) and a two-channel gated photon counter (Stanford Research Systems, model SR400). Operation of the photon counter was controlled by an AT personal computer with Stanford Research SR465 software. Spectral data were generated in binary code and converted to ASCII for processing in standard graphics software. The 488-nm line of an argon-ion laser (Coherent, model Innova 70-5) was used, and the laser power at the windowless flow cell was approximately 400 mW. A 25-cm focal length glass lens focused the laser beam onto the cell.

The liquid chromatography system consisted of an isocratic pump (Shimadzu model LC-6A) with a Rheodyne injection valve using a 20-μl sample loop. The analytical column was a 5-μm OD-MP Spheri-5 RP-18 (10 cm x 4.6 mm i.d.; Brownlee Labs). A model SPD-6A variable-wavelength UV–visible detector and a Chromatopac integrator, both from Shimadzu, were also used. The wavelength for UV detection was 254 nm. The mobile phase was methanol–water (1:1, v/v). Prior to use, the mobile phase was vigorously degassed and particulate matter was removed by passage through a 0.45-μm nylon membrane filter under vacuum. The flow rate was 0.2 ml/min. For flow injection analysis the Ag sol and drug solutions were pumped by two peristaltic pumps (Heidelberg model 131900 and Gilson model M312, respectively). The flow rate was 1.5 ml/min for silver hydrosol and 0.075 ml/min for the analyte sample.

The windowless flow cell was designed and constructed in our laboratory and consisted of two chromatographic stainless steel tubes of \( \frac{1}{16} \) inch i.d. and 1.4 mm external diameter mounted on an aluminium frame. The dimensions of the cell are shown in Fig. 2. The eluent from the chromatographic column and the silver hydrosol are mixed on a chromatographic tee and then flow to the windowless cell. The draining liquid is supported by surface tension between the two tubes. The column of liquid is approximately cylindrical with a diameter of 1.4 mm and height of 1.6 mm. The dead volume of the cell is roughly 2.5 μl. The flowing liquid exits the flow cell through the stainless steel tubing to a waste beaker.
Chemicals and procedure

All chemicals used were of analytical reagent grade or equivalent. Demineralized water was used throughout. Amiloride, amiphenazole, caffeine, chlorothalidone, hydrochlorothiazide, 2-mercaptopyridine, pemoline and triamterene were purchased from Sigma. They were used without further purification. Stock solutions (100 μg/ml) of the drugs were prepared in methanol-water (1:1, v/v). Silver hydrosols were prepared with fresh aqueous solutions of 1 x 10^{-3}M silver nitrate and 2 x 10^{-3}M sodium tetrahydroborate. Fifty millilitres of the silver nitrate solution were added dropwise to 150 ml of vigorously stirred, ice-cooled sodium tetrahydroborate solution. After mixing, the resulting colloid was maintained in an ice bath for 2 hr with constant stirring. With this procedure, the aggregation process of hydrosol has been stopped and the hydrosol remained stable for several days. Silver hydrosols were used at room temperature. Urine samples were obtained from healthy human volunteers not receiving any medical treatment and were filtered through a 0.45-μm membrane filter prior to injection.

RESULTS AND DISCUSSION

For SERS to be used as a detector for HPLC and FIA it is necessary that the analyte-induced aggregation kinetics of the colloid be rapid and that the solvent employed be adequate. Montes et al. demonstrated that the kinetics of aggregation depend on the chemical structure of the analyte studied. On the other hand, it is also known that the adsorptive behaviour of the analyte on the Ag surface can change when different types of mobile phase are used. One effect is the direct adsorption of the mobile phase onto the active sites of the colloidal particles, which diminishes the analyte's affinity...
Table 1. Surface-enhanced Raman bands of drugs on colloidal silver. Band positions are given in cm⁻¹. The values of parentheses represent the relative intensities of the bands on a semi-quantitative scale from 1 to 10.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amiloride</th>
<th>Amiphenazole</th>
<th>2-Mercaptopyridine</th>
<th>Pemoline</th>
<th>Triamterene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1082 (3)</td>
<td>1013 (8)</td>
<td>1010 (10)</td>
<td>918 (2)</td>
<td>1040 (1)</td>
<td></td>
</tr>
<tr>
<td>1209 (9)</td>
<td>1187 (6)</td>
<td>1134 (4)</td>
<td>998 (10)</td>
<td>1294 (5)</td>
<td></td>
</tr>
<tr>
<td>1375 (10)</td>
<td>1272 (5)</td>
<td>1246 (3)</td>
<td>1146 (3)</td>
<td>1357 (10)</td>
<td></td>
</tr>
<tr>
<td>1466 (8)</td>
<td>1404 (5)</td>
<td>1448 (2)</td>
<td>1246 (5)</td>
<td>1426 (5)</td>
<td></td>
</tr>
<tr>
<td>1645 (7)</td>
<td>1495 (2)</td>
<td>1570 (4)</td>
<td>1294 (5)</td>
<td>1516 (4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1606 (10)</td>
<td></td>
<td></td>
<td>1407 (6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1484 (1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1602 (8)</td>
<td></td>
</tr>
</tbody>
</table>

for the Ag surface or affects the aggregation behaviour of the Ag colloid. In the present paper, a methanol–water (1:1, v/v) mobile phase proved adequate for the drugs of interest. On the other hand, the good optical alignment of the laser beam onto the SERS cell is a critical parameter for obtaining high-quality SERS data, because the optical path length has been shown to significantly affect the SERS measurements.¹⁷

In a previous work, we have studied the SERS activity on colloidal silver of a variety of drugs.¹⁸ Five drugs have been chosen to evaluate the windowless flow cell, which provides aggregation kinetics fast enough to be processed in flow systems. Figure 3 illustrates the SER spectra of five drugs (amiloride, amiphenazole, 2-mercaptopyridine, pemoline and triamterene) obtained at room temperature by continuously flowing the analyte solution and the silver colloid through the windowless cell. The flow rates were optimized in order to maximize the signal intensity. The optimal flow rates of silver hydrosol and analyte were 1.5 and 0.075 ml/min, respectively. Few authors have reported SER spectra under flowing conditions.¹⁸¹⁹ The SER spectra in Fig. 3 demonstrate that the Raman bands are easily discernible, and each individual SER...
Table 2. Chromatographic parameters and analytical figures of merit obtained for 2-mercaptopyridine and pemoline by UV and SERS detection

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$t^*$ (min)</th>
<th>$N^*$† (µg/ml)</th>
<th>RSD§ (%)</th>
<th>LOD</th>
<th></th>
<th>(ng)</th>
<th>$t^*$ (min)</th>
<th>$N^*$† (µg/ml)</th>
<th>RSD§ (%)</th>
<th>LOD</th>
<th></th>
<th>(ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercaptopyridine</td>
<td>5.81</td>
<td>2800</td>
<td>100</td>
<td>1.9</td>
<td>0.12</td>
<td></td>
<td>5.60</td>
<td>860</td>
<td>25</td>
<td>2.5</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Pemoline</td>
<td>7.47</td>
<td>3730</td>
<td>100</td>
<td>2.5</td>
<td>1.00</td>
<td></td>
<td>7.25</td>
<td>2000</td>
<td>25</td>
<td>1.6</td>
<td>32</td>
<td></td>
</tr>
</tbody>
</table>

*Retention time obtained at a flow rate of 0.2 ml/min.
†Efficiencies in theoretical plates calculated using $N = 4 \left( \frac{t}{W_{60\%}} \right)^2$ where $W_{60\%}$ is the peak width at 60% of the peak height expressed in time units.
‡Limit for the range of linear response of peak height with concentration.
§Relative standard deviation for five replicate measurements at 25 µg/ml.
¶Limit of detection calculated for a signal equivalent to twice the baseline noise, expressed in ng injected.

The spectrum can serve as a fingerprint for each compound. Table 1 summarizes the SER band positions of the drugs.

It is important to note that the preparation of silver hydrosol affects the state of aggregation and consequently the reproducibility of results and signal stability. To eliminate this problem, silver hydrosols have to be prepared using consistent laboratory practices, as described in the Experimental section. With the procedure used, the aggregation process prior to analyte injection can be controlled. Figure 4 shows the signal reproducibility for five consecutive injections of amiloride. The absolute amount in each injection was 625 ng amiloride and the samples were injected at 6 min intervals. A band at 1375 cm$^{-1}$ in amiloride was used to monitor the signal. The measured reproducibility was 1% (relative standard deviation). Similar reproducibility was reported by Freeman et al.,$^5$ for pararosaniline hydrochloride. Figure 4 also shows the rapid baseline restoration after each injection and the absence of memory effects.

The use of the windowless flow cell for SERS detection in HPLC is illustrated in Fig. 5. An SERS-detected chromatogram of a mixture of amiloride, 2-mercaptopyridine and pemoline and a UV chromatogram of the same mixture are shown. The HPLC flow rate was 0.2 ml/min. However, the mixing of the silver sol with the column eluent just prior to the SERS detector causes the retention time to be shifted to shorter times by about 20 sec over the UV chromatogram. The relative peak areas differ on going the SERS to the UV detection modes. For instance, pemoline is the largest peak in the SERS chromatogram while 2-mercaptopyridine is the largest peak in the UV chromatogram.

A comparison of efficiency, linear range, precision and detection power for 2-mercaptopyridine and pemoline using UV detection and SERS detection is summarized in Table 2. The lower efficiency of the SERS detection results from the mixing of the eluent with the silver sol.
The precision of SERS intensities has been one of the weakest points of this technique. Remarkably, with the windowless flow cell used here, RSD values for the SERS detection mode are comparable to those found by UV detection. On the other hand, the SERS mode provides higher limits of detection. The main reason for this effect is the dilution of the analyte resulting from mixing the chromatographic eluent with the silver colloid. At the flow rates used, the dilution factor is about 20-fold. The unique selectivity provided by the SERS detector can be illustrated by comparison of chromatograms of a mixture of caffeine, chlorothalidone, hydrochlorothiazide, 2-mercaptopyridine and pemoline. Figure 6 shows the UV and SERS chromatograms of the mixture monitored at 254 nm and at 1407 cm\(^{-1}\) respectively. Caffeine and chlorothalidone co-elute and appear as a single peak in the UV chromatogram (\(t_c = 8.6\) min), while hydrochlorothiazide (\(t_c = 5.42\) min) overlaps the peak of 2-mercaptopyridine (\(t_c = 5.85\) min). The SERS-detected chromatogram presents only two peaks, at 5.6 and 7.25 min, corresponding to 2-mercaptopyridine and pemoline, respectively. Caffeine, chlorothalidone and hydrochlorothiazide are not detected since these drugs are not SERS active under the present conditions. No peak tailing or memory effects in the cell are observed. To further evaluate the selectivity of the SERS detector, an SERS chromatogram of human urine is compared with a UV chromatogram in Fig. 7. The peaks observed correspond to proteinaceous and other endogenous urine components. As shown, the peak at 7.5 min in the SERS chromatogram does not appear in the UV chromatogram. This fact indicates that there are urine components that can only be detected by SERS. On the other hand, the relative peak areas are also different in both chromatograms. For instance, the peak at 3.8 min is much higher in area in UV than SERS. The opposite is seen for the peak at 4.5 min.

CONCLUSIONS

There are advantages in using the windowless flow cell for SERS detection in HPLC. First, the open character of the cell provides continuous renewal of the aggregated colloid in the cell. This helps to remedy a recurring problem in conventional flow cell experiments, which is adsorption of aggregated silver on the cell walls resulting in undesirable memory effects. Also, baseline drifts and peak tailing are circumvented using this approach. The LODs achievable are within the limits reported for conventional batch systems, although they are much higher than in UV for two drugs studied. The unique band position and shape of spectral features in the SER spectra of the drugs could be beneficial in the dope analysis of these and similar compounds. This information could complement or supplement general gas chromatography–mass spectrometry data in confirmatory analysis. Presently, we cannot fully exploit the spectral selectivity of the SERS–HPLC approach because a monochannel detector is used to disperse the Raman scatter. Detection based on
charge-coupled devices or diode arrays will allow further refinement and evaluation of
the technique. These modifications have been initiated.

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