

# Analysis of 5-fluorouracil in saliva using surface-enhanced Raman spectroscopy

Stuart Farquharson,\* Chetan Shende, Frank E. Inscore, Paul Maksymiuk and Alan Gift

Real-Time Analyzers, Inc., 87 Church Street, East Hartford, Connecticut 06108, USA

Received 1 July 2004; Accepted 23 September 2004

The ability of surface-enhanced Raman spectroscopy (SERS) to measure the chemotherapy drug 5-fluorouracil in saliva is presented. A silver-doped sol-gel provided SERS and also some chemical selectivity. 5-Fluorouracil and physiological thiocyanate produced SERS, whereas large biochemicals, such as enzymes and proteins, did not, supporting the expectation that the larger molecules do not diffuse through the sol-gel to any appreciable extent. In addition, 5-fluorouracil samples of  $2 \mu\text{g ml}^{-1}$  were easily measured, and an estimated limit of detection of  $150 \text{ ng ml}^{-1}$  in 5 min should provide sufficient sensitivity to perform pharmacokinetic studies and to monitor and regulate patient dosage. This would fill a critical need for this highly used drug, since genetic-based variations in its metabolism can range by as much as five-fold from one patient to another. Copyright © 2004 John Wiley & Sons, Ltd.

**KEYWORDS:** chemotherapy drugs; drug dosage; drug metabolism; surface-enhanced Raman spectroscopy

## INTRODUCTION

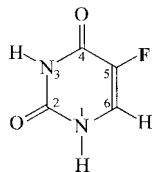
Determining safe and effective dosages for chemotherapy drugs remains a significant challenge in the treatment of cancer.<sup>1,2</sup> Many chemotherapy drugs, such as 5-fluorouracil, prevent cell growth through their incorporation into DNA and RNA, which causes improper replication and incomplete biochemical synthesis. Their success is based on the higher rate of cancer cell multiplication than normal cells. Nevertheless, normal cell growth is also adversely affected, especially those that also involve rapid cell turnover, such as blood cell production in bone marrow. These dangerous side-effects preclude the use of clinical trials to establish a statistical basis for dosage, and the latter is based on the limited set of previously treated patients. A secondary approach to determining safe and effective dosage is to monitor its metabolism during administration and adjust concentrations accordingly. This information can be extremely beneficial since the patient's genetic makeup and nutritional habits can strongly influence the pharmacokinetics of these drugs.<sup>3</sup> Unfortunately, current practices require 10–20 ml of blood per analysis,<sup>4</sup> and the multiple samples required to profile pharmacokinetics may further jeopardize the patient's health and, consequently, are rarely performed.<sup>4,5</sup>

Saliva analysis has long been considered an attractive alternative, and recent research has shown that drug metabolism is often equally represented in saliva as in blood plasma,<sup>6–9</sup> typically at  $\mu\text{g ml}^{-1}$  concentrations. Analysis of saliva is highly desirable in that it is non-invasive, reduces the risk of HIV infection, is easier to obtain and is easier to analyze chemically. Saliva is 99.5% water, and the concentrations of interfering physiological chemicals are typically 100 or more times lower than in blood plasma or urine.<sup>10</sup> Unfortunately, current saliva analysis, like blood, requires 10–20 ml of sample to separate and detect drugs and their metabolites chemically, and this quantity is difficult to generate.

In an effort to overcome this limitation, we have been investigating the potential of surface-enhanced Raman scattering (SERS) to perform the needed measurements in saliva. The rich molecular information provided by Raman scattering and the increased sensitivity afforded by surface enhancement from silver or gold nanoparticles has been successfully used to measure numerous drugs,<sup>11–16</sup> and it may be possible to develop a simple SERS-active device to analyze drugs and metabolites in <1 ml of saliva in <5 min.

Here we examined this potential by analyzing 5-fluorouracil (5-FU, Fig. 1) in saliva. 5-FU is one of the most often used drugs to treat solid tumors and colorectal carcinoma.<sup>17</sup> The metabolism, mechanisms of action and chemotherapeutic usage of 5-FU have recently been reviewed.<sup>17</sup> In addition to being converted to one of three active forms, 5-FU is also converted to an inactive form,

\*Correspondence to: Stuart Farquharson, Real-Time Analyzers, Inc., 87 Church Street, East Hartford, Connecticut 06108, USA.  
E-mail: stu@rta.biz  
Contract/grant sponsor: National Institutes of Health;  
Contract/grant number: 1R43CA94457-01.



**Figure 1.** Structure of 5-fluorouracil, with standard numbering.

dihydrofluorouracil (5-FUH<sub>2</sub>) by dihydropyrimidine dehydrogenase (DPD). There is a wide genetic-based variation in the amount of DPD in individuals and the amount of 5-FU converted to 5-FUH<sub>2</sub> can range from 15 to 80%. Consequently, employing a 'standard' dose of 5-FU has led to severe toxicity or even death in individuals deficient in DPD.<sup>18</sup>

A recent pharmacokinetic investigation has shown that 5-FU concentrations in saliva closely match those in plasma, reaching a maximum of 15–28  $\mu\text{g ml}^{-1}$  and a 3 h minimum of 0.1  $\mu\text{g ml}^{-1}$  (patient dependent).<sup>9</sup> Here we present preliminary data supporting the concept that near continuous monitoring of drug and metabolite concentrations afforded by SERS of saliva would allow more effective control of dosage and thereby minimize toxic effects on a patient-by-patient basis.

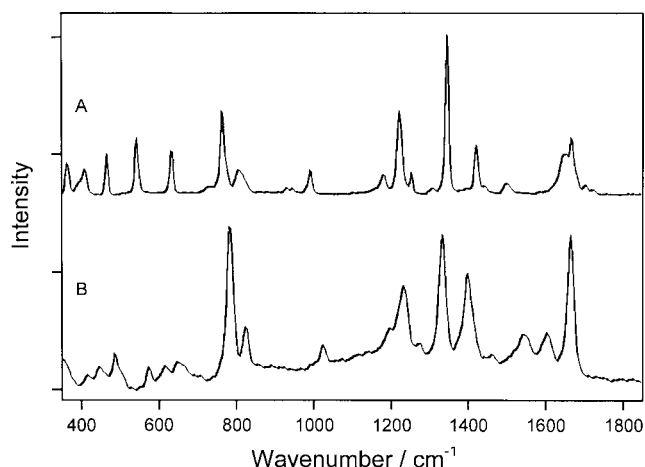
## EXPERIMENTAL

5-Fluorouracil, sodium thiocyanate and all chemicals used to prepare the silver-doped sol–gel-coated vials were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. HPLC-grade water was used to prepare samples ranging in concentration from 2 to 1000  $\mu\text{g ml}^{-1}$ . SERS-active vials were prepared according to published procedures,<sup>19</sup> using a silver amine precursor to provide the metal dopant and an alkoxide precursor to provide the sol–gel matrix. The silver amine precursor consisted of a 5:1 (v/v) ratio of 1 N  $\text{AgNO}_3$  to 28%  $\text{NH}_4\text{OH}$ , and the alkoxide precursor consisted of a 2:1 (v/v) ratio of methanol to tetramethyl orthosilicate. The alkoxide and silver amine precursors were mixed in an 8:1 (v/v) ratio, then 140  $\mu\text{l}$  were introduced into 2 ml glass vials, which were spin-coated. After sol–gel formation, the incorporated silver ions were reduced with dilute sodium borohydride. For the pH study of 5-FU, a 1 mg  $\text{ml}^{-1}$  stock solution was prepared, adjusted to pH 3.7 using 0.1 M  $\text{HNO}_3$ , and then added to a 2 ml SERS-active vial. After the SERS measurement, the 2 ml of solution was returned to the stock solution and made basic using 0.1 M KOH. Prior to re-addition to the same SERS-active vial, the vial was first rinsed three times with distilled water, then twice with the new solution prior to SERS measurement. This procedure was repeated until a pH of 10.7 was obtained and measured. Then the entire sequence was repeated, but instead 0.1 M  $\text{HNO}_3$  was used to make the solution incrementally acidic to a final measurement pH of 3.3. In all, pH measurements were performed at 3.3, 3.7, 4.3, 4.8, 5.4, 5.6, 6.5, 9.2 and 10.7.

The vials were mounted horizontally on an XY positioning stage (Conix Research, Springfield, OR, USA), such that the focal point of an  $f/0.7$  aspheric lens was just inside the glass wall. The lens focused the beam into the sample and collected the scattered radiation back along the same axis. A dichroic filter (Omega Optical, Brattleborough, VT, USA) was used to reflect the excitation laser to the lens and pass the Raman scattered radiation collected by the lens. An  $f/2$  achromat was used to collimate the laser beam exiting a 200  $\mu\text{m}$  core diameter source fiber optic, and a second  $f/2$  achromat was used to focus the scattered radiation into a 365  $\mu\text{m}$  fiber optic (Spectran, Avon, CT, USA). A short-pass filter was placed in the excitation beam path to block the silicon Raman scattering generated in the source fiber from reflecting off sampling optics and reaching the detector. A long-pass filter was placed in the collection beam path to block the sample Rayleigh scattering from reaching the detector. A 785 nm diode laser (Model 785-600, Process Instruments, Salt Lake City, UT, USA) was used to deliver 100–150 mW of power to the sample [up to 400 mW for normal Raman scattering (RS)]. A Fourier transform Raman spectrometer (Model IRA-785, Real-Time Analyzers, East Hartford, CT, USA) equipped with a silicon photo-avalanche detector (Model C30902S, Perkin-Elmer, Stamford, CT, USA) was used to acquire the SERS and RS spectra. All spectra were collected at 8  $\text{cm}^{-1}$  resolution. For each sample, nine spectra were recorded along the length of the vial with 1 mm spacing. As a practical approach to minimizing the variability associated with the SERS activity as a function of sample position, three high- and three low-intensity spectra were discarded, while the three median spectra were averaged and reported. Relative standard deviations for all concentrations reported are included in figures as  $\pm$  error bars. All peak intensities used for calculations were measured peak heights.

## RESULTS AND DISCUSSION

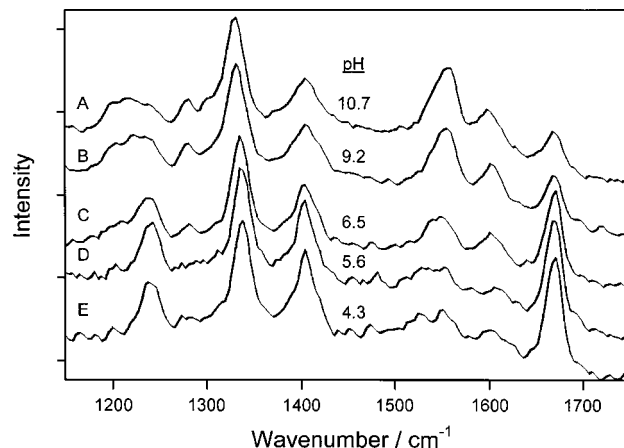
SERS spectra of chemicals are often different than their normal Raman spectral counterparts. This is largely due to surface interactions that enhance various vibrational modes to different extents. Further, it has been shown that a layer of negative charge on silver provided by chloride anions can greatly enhance the interaction of some analytes and substantially increase SERS.<sup>20,21</sup> In fact, the SERS of uracil in 0.1 M KCl obtained on a silver electrode was found to be substantially more intense than uracil obtained on silver embedded in a sol–gel.<sup>22</sup> Here, the silver-doped sol–gels were rinsed with 5 mM HCl after reduction to provide a negatively charged layer and improve the SERS activity. The RS and SERS spectra of 5-FU are compared in Fig. 2 for a crystalline powder and 1 mg  $\text{ml}^{-1}$  water, respectively. Both spectra, not surprisingly, are very similar to the corresponding spectra obtained for uracil,<sup>22–25</sup> and the peaks can be assigned to vibrational modes accordingly. The



**Figure 2.** (A) Raman and (B) surface-enhanced Raman spectra of 5-fluorouracil. Conditions: (A) crystalline powder, 150 mW of 785 nm, 1 min; (B) 1 mg ml<sup>-1</sup>, 100 mW of 785 nm, 2 min.

Raman spectrum [Fig. 2(A)] is dominated by peaks at 766, 1223, 1347, 1423 cm<sup>-1</sup>, and a doublet at 1653 and 1669 cm<sup>-1</sup>. These peaks have been assigned to the pyrimidine ring-breathing mode, a concerted ring mode, an unassigned mode, NH in-plane deformations and the two carbonyl stretches (C=O), respectively.<sup>23,24</sup> In the SERS spectrum [Fig. 2(B)], these peaks shift to 786, 1234, 1335, 1400 and 1667 cm<sup>-1</sup>. Only one carbonyl peak is observed, suggesting a preferred orientation. The combination of the attractive forces between the chloride anion surface layer and the nitrogen protons and the repulsive forces between the fluorine and the same layer favors orientation with C<sub>4</sub>=O towards the surface, and it is likely that it is this carbonyl mode that is enhanced. The wavenumber at which this SERS peak occurs is nearly the same as the higher wavenumber carbonyl peak in the RS that is assigned to the C<sub>4</sub>=O mode, supporting this reasoning.<sup>23</sup> The intensity of the SERS carbonyl mode and the shift in wavenumber by as much as 20 cm<sup>-1</sup> for other modes suggest a strong surface interaction. A similar conclusion was drawn for uracil based on the observed shift of the ring-breathing mode from 780 to 802 cm<sup>-1</sup>.<sup>25</sup>

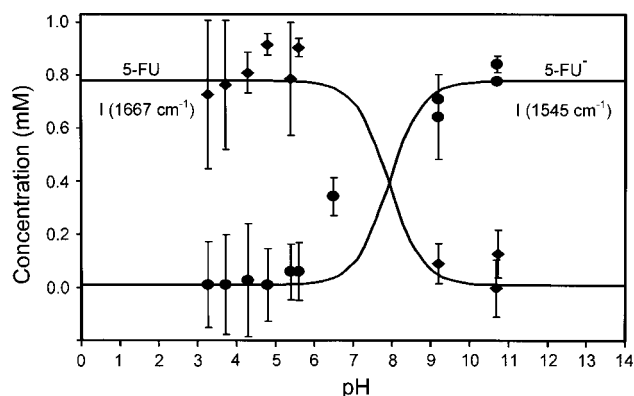
5-FU is a weak acid with a pK<sub>a</sub> of 7.93,<sup>26</sup> and consequently, depending on the ionic form, the pH can also strongly influence the molecule to surface interaction.<sup>27</sup> The pH dependence of the SERS spectrum is essential if quantitative measurements are to be made. Figure 3 shows the SERS spectra of 5-FU for selected pH values of 4.3, 5.6, 6.5, 9.2 and 10.7. The peaks at 1234, 1400 and 1667 cm<sup>-1</sup> decrease in intensity whereas those at 786 and 1335 cm<sup>-1</sup> remain constant as the pH is made basic. Also, the last two peaks both shift in wavenumber towards each other by ca 8 cm<sup>-1</sup> as the pH is changed from 3.3 to 10.7. Although this shift appears to follow the trend in pH, it is equivalent to the spectral resolution of the measurements and was not further analyzed. In addition, two new peaks appear at 1545 and



**Figure 3.** (A) Surface-enhanced Raman spectra of 1 mg ml<sup>-1</sup> 5-FU in water at pH (A) 10.7, (B) 9.2, (C) 6.5, (D) 5.6 and (E) 4.3. Conditions: 100 mW of 785 nm, 2 min.

1606 cm<sup>-1</sup>. The decrease in the intensity of the 1234, 1400 and 1667 cm<sup>-1</sup> peaks is a consequence of the anion being formed. Deprotonation occurs at the N<sub>3</sub> position,<sup>26</sup> which essentially removes this interaction with the chloride anions on the silver surface, and also that of the nearby carbonyl groups. It also appears that the intensity of the 1400 cm<sup>-1</sup> NH deformation is reduced by ~50%, consistent with elimination of one of the two modes. The appearance of the peak at 1545 cm<sup>-1</sup>, assigned to the C<sub>6</sub>-H deformation mode, suggests that this part of the molecule now interacts with the chloride anion coated surface. The 1606 cm<sup>-1</sup> peak is tentatively assigned to a trigonal ring mode, based on the same mode observed in the SERS spectra of pyrazine at 1590 cm<sup>-1</sup>,<sup>28</sup> and the fact that formation of the anion leads to a high degree of  $\pi$ -electron delocalization.<sup>26</sup>

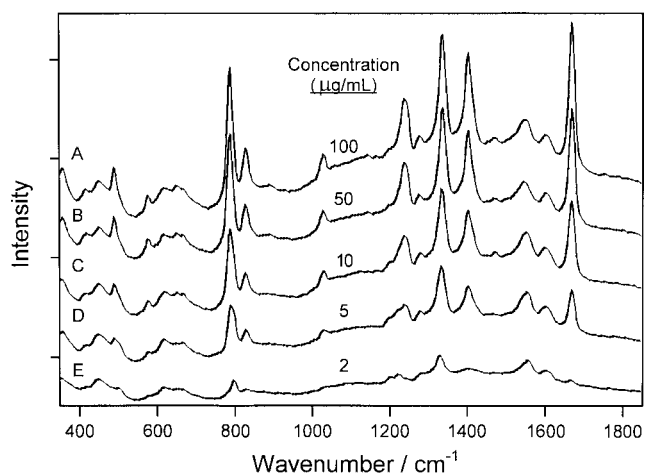
The intensities of the 1545 and 1667 cm<sup>-1</sup> peaks, normalized to the 1335 cm<sup>-1</sup> peak intensity, are plotted in Fig. 4



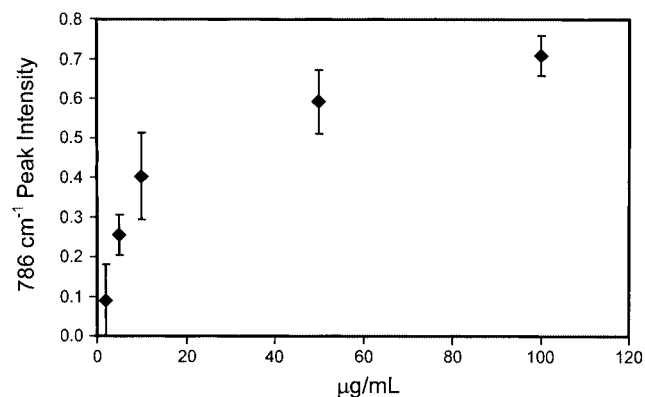
**Figure 4.** Plot of the 1545 (●) and 1667 cm<sup>-1</sup> (◆) peak intensities (heights normalized to the 1335 cm<sup>-1</sup> peak height) as a function of pH representing 5-FU<sup>-</sup> and 5-FU, respectively. Lines represent predicted relative concentrations based on the pK<sub>a</sub> of 7.93.

as a function of pH and compared with the calculated concentration of the neutral and anionic forms based on the  $pK_a$ . The decrease in the latter peak and increase in the former peak coincident with the  $pK_a$  supports their respective assignment to the neutral and anionic forms. It is important to note that the 786 and 1335  $\text{cm}^{-1}$  peak intensities are nearly constant as a function of pH and are therefore appropriate for quantitative analysis.

A preliminary calibration curve relating SERS intensity to concentration was prepared by measuring 100, 50, 10, 5 and 2  $\mu\text{g ml}^{-1}$  samples (Fig. 5). The SERS intensity was taken as the peak height at 786  $\text{cm}^{-1}$  minus the value at 750  $\text{cm}^{-1}$  as the baseline. This peak intensity increases rapidly with concentration to 5–10  $\mu\text{g ml}^{-1}$  and then levels off (Fig. 6). This Langmuir isotherm response is typical for SERS substrates where signal intensity is a function of available silver surface area.<sup>29</sup> The 'roll-over' concentration represents the point at which monolayer coverage has been reached, and higher concentrations do not produce significant increases



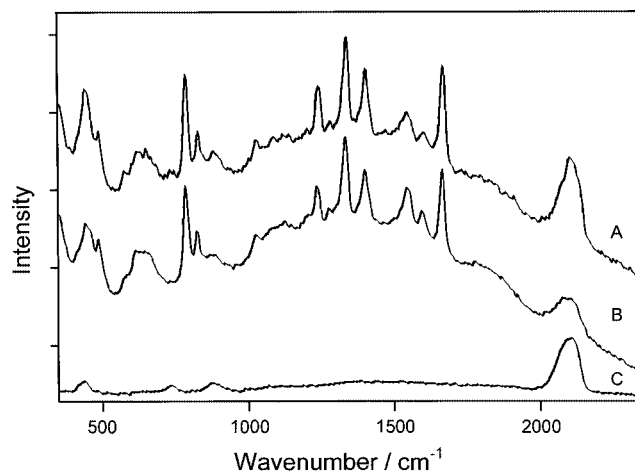
**Figure 5.** Surface-enhanced Raman spectra of 5-FU for concentrations of (A) 100, (B) 50, (C) 10, (D) 5 and (E) 2  $\mu\text{g ml}^{-1}$ . Conditions: 100 mW of 785 nm, 5 min.



**Figure 6.** Surface-enhanced Raman spectral response as a function of 5-FU concentration. Conditions as in Fig. 5.

in intensity. It can be seen that even at 2  $\mu\text{g ml}^{-1}$  [Fig. 5(E)], the signal-to-noise quality is quite good. This spectrum was used to estimate a limit of detection (LOD), defined as the concentration that produces a peak three times as intense as the baseline noise. Using the intensity of the 786  $\text{cm}^{-1}$  peak, and the relative standard deviation of baseline noise, an LOD of 150  $\text{ng ml}^{-1}$  is estimated for 100 mW of 785 nm laser power at the sample and an integration time of 5 min.

As a preliminary demonstration of the proposed application, several samples were prepared by adding 500  $\mu\text{g}$  of 5-FU to 1 ml of saliva and the SERS spectra were recorded (Fig. 7). In all cases, the 5-FU peaks were readily apparent, indicating that this chemotherapy drug could be identified in saliva. The average 786  $\text{cm}^{-1}$  peak height for each sample ranged from 0.65 to 0.8, which corresponds to the monolayer regime according to Fig 6, and accurate determination of concentration is not possible. Nevertheless, these values are not inconsistent with the initial concentration. For several samples two new peaks appeared at 445 and 2095  $\text{cm}^{-1}$ . The latter peak is characteristic of cyanide and thiocyanate chemical species. Cyanide has been observed in the saliva of cigarette smokers,<sup>30</sup> and thiocyanate is observed in most people, likely as an antibacterial agent.<sup>31</sup> The two peaks are assigned to thiocyanate based on previous SERS spectra recorded using silver electrodes.<sup>32</sup> This was confirmed by preparing and measuring SERS spectra of this chemical with the silver-doped sol-gels [Fig. 7(C)]. The three thiocyanate peaks are the N–C–S bend at 445  $\text{cm}^{-1}$ , the C–S stretch at 735  $\text{cm}^{-1}$ , and the C–N stretch at 2095  $\text{cm}^{-1}$ . The 879  $\text{cm}^{-1}$  peak is attributed to trace amounts of boric acid generated by the silver reduction process.<sup>33</sup> Although these peaks do not interfere spectrally with the characteristic 5-FU peaks, the thiocyanate could block the SERS-generating metal surfaces. The large biochemicals of saliva, such as  $\alpha$ -amylase



**Figure 7.** Surface-enhanced Raman spectra of 0.5 mg of 5-FU added to 1 ml of saliva: (A) sample 1 and (B) sample 2. (C) 1  $\text{mg ml}^{-1}$  KSCN in water for comparison. Conditions: 100 mW of 785 nm, 1 min.

and mucin, represent a similar concern. However, no peaks appear that suggest that they are present on the silver particles. It is possible that these molecules are SERS inactive, but more likely they probably simply do not diffuse through the sol-gel pores.

## CONCLUSION

The silver-doped sol-gels used in this study provided good-quality SERS spectra of 5-fluorouracil and some chemical separation from saliva. The unique SERS peaks were all easily observed at  $2 \mu\text{g ml}^{-1}$ . Measurements of 5-FU artificially added to real saliva samples also provided high-quality spectra, but physiological thiocyanate was observed in some samples. The sol-gels appear selectively to allow smaller molecules, such as 5-FU and thiocyanate, to diffuse through to the silver particles, but exclude many large saliva biochemicals. The estimated limit of detection for 5-FU of  $150 \text{ ng ml}^{-1}$  is within the  $1\text{--}30 \mu\text{g ml}^{-1}$  expected range of physiological concentrations. This should provide ample sensitivity to perform pharmacokinetic studies and to monitor and regulate patient dosage. Current work involves improving the reproducibility and expanding the capabilities to include the measurement of 5-FU<sub>2</sub> and leucovorin. The latter is a drug often included with 5-FU treatment to reduce deleterious side-effects. We believe that this preliminary work has demonstrated the potential of developing a simple saliva sample system, based on a SERS-active sol-gel, which may have great value in monitoring and regulating chemotherapy drug dosage. Such a device may be broadly applicable to monitoring other drugs as they are evaluated during clinical trials.

## Acknowledgements

The authors are grateful to Dr John Murren of Yale University for providing important insight into the process of administering chemotherapy drugs. Dr Farquharson acknowledges his late sister Beth Ordway, who donated blood samples during her struggle against cancer, to further the understanding of chemotherapy drug pharmacokinetics. This work was supported by the NIH through grant number 1R43CA94457-01.

## REFERENCES

- Ames BN, Gold LS, Willet WC. *Proc. Natl. Acad. Sci. USA* 1995; **90**: 7915.
- Dollinger M, Rosenbaum EH, Cable G. *Everyone's Guide to Cancer Therapy*. Somerville House Books: Kansas City, 1994.
- Ames BN, *Toxicol. Lett.* 1998; **102–103**: 5.
- Goodman M, Riley MB. In *Cancer Nursing: Principles and Practice* (4th edn), Groenwald SL, Frogge MH, Goodman G, Yarbrow CH (eds). Jones and Bartlett: Boston, 317–386; 1997.
- Cone ET, Jenkins AJ. In *Handbook of Analytical Therapeutic Drug Monitoring and Toxicology*, Wong SHY, Sunshine I (eds). CRC Press: New York, 1997; Chapt. 18.
- van Warmerdam LJ, van Tellingen O, ten Bokkel-Huinink WW, Rodenhuis S, Maes RA, Bijnen JH. *Ther. Drug Monit.* 1995; **17**: 465.
- Takahashi T, Fujiwara Y, Sumiyoshi H, Isobe T, Yamaoka N, Yamakido M. *Cancer Chemother. Pharmacol.* 1997; **40**: 449.
- de Jonge MJ, JV Verwiej, Loos WJ, Dallaire BK, Sparreboom A. *Clin. Pharmacol. Ther.* 1999; **65**: 491.
- Joulia JM, Pinguet F, Ychou M, Duffour J, Astre C, Bressolle F. *Eur. J. Cancer* 1999; **35**: 296.
- Chamberlain J. *The Analysis of Drugs in Biological Fluids* (2nd edn). CRC Press: New York, 1995.
- Sutherland WS, Laserna JJ, Angebrannt MJ, Winefordner JD. *Anal. Chem.* 1990; **62**: 689.
- Nabiev I, Baranov A, Chourpa I, Beljebbar A, Sockalingum G, Manfait M. *J. Phys. Chem.* 1995; **99**: 1608.
- Perez R, Ruperez A, Laserna JJ. *Anal. Chim. Acta* 1998; **76**: 255.
- Beljebbar A, Sockalingum G, Morjani H, Manfait M. *Proc. SPIE* 1999; **3608**: 175.
- Farquharson S, Lee YH. *Proc. SPIE* 2000; **4200**: 89.
- Gift A, Shende C, Inscore F, Maksymiuk P, Farquharson S. *Proc. SPIE* 2004; **5261**: 135.
- Malet-Martino M, Martino R. *T. Oncologist* 2002; **7**: 288.
- Diasio RB, Beavers TL, Carpenter JT. *J. Clin. Invest.* 1988; **81**: 47.
- Farquharson S, Maksymiuk P. *Appl. Spectrosc.* 2003; **57**: 479.
- Jeanmaire D, van Duyne RP. *J. Electroanal. Chem.* 1977; **84**: 1.
- Dou X, Jung YM, Cao X-Q, Ozaki Y. *Appl. Spectrosc.* 1999; **53**: 1440.
- Farquharson S, Smith WW, Lee YH, Elliott S, Sperry JF. *Proc. SPIE* 2002; **4575**: 62.
- Lord RC, Thomas GJ Jr. *Spectrochim. Acta* 1967; **23**: 2551.
- Tsuboi M, Takahashi S, Harada I. In *Physico-Chemical Properties of Nucleic Acids*, vol. 2, Duchesne J (ed). Academic Press: New York, 1973; Chapt. 11.
- Suh JS, Moskovits M. *J. Am. Chem. Soc.* 1986; **108**: 4711.
- Jang YH, Sowers LC, Gagin T, Goddard WA III. *J. Phys. Chem. A* 2001; **105**: 274.
- Farquharson S, Gift A, Maksymiuk P, Inscore F, Smith W. *Proc. SPIE* 2004; **5269**: 117.
- Farquharson S, Lay PA, Weaver MJ. *Spectrochim. Acta, Part A* 1984; **40**: 907.
- Mullen K, Carron K. *Anal. Chem.* 1994; **66**: 478.
- Tsuge K, Kataoka M, Seto Y. *J. Health Sci.* 2000; **46**: 343.
- Ermans AM, Delange F, van der Velden M, Kinthaert J. In *Human Development and the Thyroid Gland. Relation to Endemic Cretinism*, Stanbury JB, Kroc RL (eds). Plenum Press: New York, 1972; 455.
- Tadayoni MA, Farquharson S, Li TT-T, Weaver MJ. *J. Phys. Chem.* 1984; **88**: 4701.
- Nyquist RA, Putzig CL, Leugers MA. *Handbook of Infrared and Raman Spectra of Inorganic Compounds and Organic Salts*, vol. 4. Academic Press: New York, 1997; 44.